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<b>(21) International Application Number:</b> PCT/US98/15067 <b>(22) International Filing Date:</b> 21 July 1998 (21.07.98) <b>(30) Priority Data:</b> 60/053,599                      21 July 1997 (21.07.97)                      US 09/119,707                      20 July 1998 (20.07.98)                      US <b>(71) Applicant (for all designated States except US):</b> CERUS CORPORATION [US/US]; 2525 Stanwell Drive, Concord, CA 94520 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GREENMAN, William, M. [US/US]; 3834 23rd Street, San Francisco, CA 94114 (US). GRASS, Joshua, A. [US/US]; 3187 Ida Drive, Concord, CA 94519 (US). TALIB, Sohel [US/US]; 404 Camberly Way, Redwood City, CA 94061 (US). STASSINOPOULOS, Adonis [GR/US]; 7065 York Court, Dublin, CA 94568 (US). HEI, Derek, J. [US/US]; 4405 Smoke Tree Court, Concord, CA 94521 (US). HEARST, John, E. [US/US]; 101 Southhampton Street, Berkeley, CA 94717 (US). <b>(74) Agents:</b> BRENNAN, Sean, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> METHOD OF TREATING LEUKOCYTES, LEUKOCYTE COMPOSITIONS AND METHODS OF USE THEREOF		
<b>(57) Abstract</b> <p>The invention provides methods and compositions for treating leukocytes to arrest proliferation of the leukocytes and render them ineffective in eliciting graft-versus-host disease (GVHD), but effective to enhance engraftment of allogeneic donor cells and promote destruction of diseased cells or pathogens. Leukocyte compositions and methods of use of these compositions in alleviating disease, facilitating various types of immune reconstitution and immunotherapy, and enhancing engraftment of allogeneic donor cells, are also provided.</p>		

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## METHOD OF TREATING LEUKOCYTES, LEUKOCYTE COMPOSITIONS AND METHODS OF USE THEREOF

### REFERENCE TO RELATED APPLICATIONS

5           This application claims priority to U.S. Provisional Patent Application Serial No. 60/053,599, filed July 21, 1997.

### FIELD OF THE INVENTION

10           The present invention relates to cell compositions that are incapable of proliferation but retain function, and the methods of preparation and use of such compositions. More specifically, the present invention relates to methods for preparing proliferation inhibited leukocytes for use in transfusion.

### BACKGROUND

15           Bone marrow transplantations (BMT) are performed to treat a variety of malignant and nonmalignant hematologic diseases, including leukemias, multiple myeloma, lymphomas, anemias, and immunodeficiency diseases such as severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome and aplastic anemia.

20           Leukemias are malignant neoplasms of hematopoietic tissues. These neoplasms are categorized into two predominant forms: chronic and acute. While acute leukemias are characterized by undifferentiated cell populations (*e.g.* Acute Lymphocytic Leukemia or "ALL" and Acute Myelogenous Leukemia or "AML"), chronic Leukemias usually present a more mature morphology (*e.g.*, Chronic Myelocytic Leukemia or "CML" and Chronic Lymphocytic Leukemia or "CLL"). In the United States in 1995, there were approximately 25,700 new cases of leukemia, of which about 4,500 were CML. In 1996, approximately 7,000 new cases of CML were diagnosed in the U.S. of which approximately 30% will ultimately receive an allo BMT (Leukemia Society: Leukemia Society Web Site, 1997). About 50% of the BMT patients are expected to suffer a relapse in the leukemia.

30           The general goal of leukemia therapy is to arrest the proliferation of abnormal morphologies and restore "normal" hematopoiesis in the bone marrow. Treatment regimens may include a combination of chemotherapy, radiation therapy, and bone marrow transplantation. In a "generic" allogeneic bone marrow transplantation (allo-

BMT) protocol, a leukemia patient is first given a myeloablative dose of chemoradiotherapy. This phase of treatment is designed to eradicate all leukemia cells and their progenitors. Having arrested the pathological hematopoietic physiology, the functionally inoperative bone marrow is subsequently replaced with allogeneic disease-free stem cells. In optimal circumstances, this transplanted marrow facilitates the restoration of normal polyclonal hematopoietic proliferation. The improvement in disease control also relies on an immune mediated response of the graft against the leukemia cells, referred to as the graft-versus-leukemia (GVL) effect.

Bone marrow is not the exclusive source of hematopoietic progenitors. Peripheral blood and cord blood may also be used as a source of stem cells. *See McCullough, The New Generation of Blood Components, Transfusion, 35:374 (1995).* Trophic factors (such as granulocyte-colony-stimulating factor) may be administered systemically to increase the proliferation of peripheral blood stem cells thereby creating alternatives to bone marrow for the harvest of hematopoietic progenitors.

Unfortunately, the major obstacles to successful BMT are Graft-versus-Host Disease (GVHD), infections and relapse of the underlying disease. GVHD and infections are responsible for 10-30% of morbidity and mortality in the first 100 days following transplantation (Basic and Clinical Immunology, 8th edition, Daniel P. Stites, Abba I. Terr and Tristram G. Parslow (eds.), Appleton & Lange, Norwalk, CT, 1994). If the BMT fails, a “relapse” can result from the unchecked proliferation of residual tumor cells. *See Slavin et al., “Immunotherapy of Minimal Residual Disease by Immunocompetent Lymphocytes and their Activation Cytokines,” Cancer Investigation, 10:221 (1992).* The medical treatment options in the past have been quite limited in such situations. For example, second bone marrow transplants and/or the extended use of cytotoxic drugs are associated with poor survival.

Nonetheless, a treatment strategy has emerged from a more complete understanding of the transplant biology. Studies on BMT with or without T-cell depletion showed that T-cells are important for the antileukemic treatment which can be useful in the management of minimal residual disease or “full blown” relapse in CML patients. With this understanding, treatment of leukemia patients with Donor Leukocyte Infusion (DLI) after bone marrow transplants has become an accepted treatment. *See Kolb et al., Blood 76:2462 (1990); Lim, S.H et al., Am. J. Hem. 54:61–67, 1997; Giralt, S.A. et al., Cur. Op. Onc. 8:96–102, 1996; Barrett, J. et al., Cur. Op. Onc. 8:89–95, 1996.* Transfusion of



leukocytes has also been used as adoptive immunotherapy after failure of bone marrow transplantation to induce permanent remission. See J. McCullough, "The New Generation of Blood Components," *Transfusion* 35:374 (1995). DLIs are particularly effective for the treatment of CML, but are also used for the treatment of AML, ALL and CLL. DLIs have also been used for the treatment of myelodysplasia (MDS), non-Hodgkin's lymphoma, Hodgkin's disease and multiple myeloma. The infused cells seem to generate an immune response against the recipient's malignant cells. Approximately 70% of the patients with CML relapsing into a chronic phase have achieved durable cytogenetic remissions after DLI and become negative for residual disease by PCR analysis for the bcr-abl rearrangement (Philadelphia chromosome)(Drobyski, W.R. *et al.*, *Blood* 82:2310–2318, 1993).

This approach has risks, however, since GVHD occurs in 80%, and marrow aplasia in 56%, of the patients. These are the two major causes of treatment failure, accounting for the deaths of up to 20% of responding patients (Champlin, R. *et al.*, *Act. Haemat.* 95:157–163, 1996). GVHD results from donor T-lymphocytes present in the transfused BMT or DLI, proliferating and colonizing host tissues. Once proliferated, the donor T-cells attack host tissues causing the pathological symptoms.

The eradication of disease after DLI is thought to be due to an immune reaction of the graft against the tumor cells, i.e., GVL effect (Barrett, J. *et al.*, *Cur. Op. Onc.* 8:89–95, 1996). The mechanism for the GVL effect is not as well understood. It is effected by T-cells derived from the donor, as T-cell depleted BMT leads to higher incidences of leukemia relapse. An immune response is necessary for the effect to occur which has been experimentally demonstrated by the higher rates of relapse following syngeneic BMTs as compared to allogeneic BMTs (Duell, T., *Ann. Int. Med.* 126:184–192, 1997). In Japan, the high homogeneity of the population has resulted in ineffective DLI therapy since the random donors and hosts are not sufficiently allogeneic (Takahashi, K. *et al.*, *Lancet.* 343:700–702, 1994).

Clinical results indicate that the GVL effect is separable from pathogenic GVHD. Patients treated with T-cell depleted allogeneic BMTs showed a higher incidence of relapse than patients which were treated with BMT and standard GVHD prophylaxis, even when controlled for the number of GVHD incidences (Horowitz, M.M. *et al.*, *Blood* 75:555–562, 1990). There are also many reported cases where remission from CML was observed without the onset of GVHD (Duell, T., *Ann. Int. Med.* 126:184–192, 1997). In

animal models the separation of GVHD from GVL is achievable (See e.g., Johnson, B.D. *et al.*, *Bone Marrow Transplant.* 11:329–336, 1993; Glass, B. *et al.*, *Br. J. Hem.* 93:412–420, 1996; Johnson, B.D. *et al.*, *Blood* 85:3302–3312, 1995).

To avoid acute GVHD, several techniques have been used to purge donor  
5 T-lymphocytes or mature lymphocytes present in the BMT. However, there is currently no effective treatment of bone marrow to completely avoid GVHD, since in most cases the GVL effect is compromised, resulting in higher relapse rates (Kantarjian, H.M. *et al.*, *Blood* 87:3069–3081, 1996). Some experimental approaches in humans and animal  
10 models involve the depletion of a T-cell subset, UVB irradiation (Greenfeld, J.I. *et al.*, *J. Sur. Res.* 60:137–141, 1996), or T-cell depletion followed by the readdition of defined number of T-cells, titrating the absence of GVHD of defined numbers of T-cells (Drobyski, W.R. *et al.*, *Blood* 82:2310–2318, 1993). In one strategy, the donor T-cells are transfected with a “suicide gene”, the herpes virus thymidine kinase (HSV-tk) gene which confers sensitivity to the drug gancyclovir. If GVHD symptoms are observed, the patient  
15 is given gancyclovir to eliminate the T-cells *in vivo*, until the symptoms regress (Bordignon, C. *et al.*, *Hum. Gen. Ther.* 6:813–819, 1995; Bonini C. *et al.* *Science* 276:1719-1724, 1997). Another “suicide gene” approach utilizes the FAS gene encoding an apoptosis signaling protein (Ariad Pharmaceuticals). In the FAS system, a dimerizing agent such as FK1012 is used to induce FAS production which kills the donor cells that  
20 express the gene.

The “suicide gene” approach, while perhaps attractive from the point of view of eliminating unwanted T-cells after residual cancer is treated, has a number of significant disadvantages. First, GVHD symptoms are used as the signal to begin treatment with the drug. Therefore, GVHD is allowed to start and must be shut down. Second, the drug used  
25 to eliminate the T-cells must be given systemically, thereby exposing the entire body to drug for the time necessary to bring GVHD under control. Gancyclovir has toxic side effects and the HSV-tk protein is immunogenic. Additionally, because the patients need to be protected from the severe forms of GVHD, immunosuppressive prophylaxis remains an absolute requirement. Also, in some instances of chronic GVHD, the administration of  
30 gancyclovir was proven not to be fully effective (Bonini C. *et al.* *Science* 276:1719-1724, 1997). This is probably due to the caveat of this approach which is that only proliferating cells will be gancyclovir sensitive. Furthermore, even proliferating cells can escape the

effect of gancyclovir through enzymatic assistance offered by neighboring normal cells present in the vicinity of the HSV-tk cells (Good Samaritan Effect).

With the FAS system, the disadvantages include the difficulty in the synthesis and the insolubility of the dimerizing agents that induce FAS production. The binding of the dimerizing agents to FKBP protein in the host may limit the available drug.

Furthermore, technical and practical concerns related to the gene therapy make the application of the technology difficult to implement and control. The production of transfected cells is plagued by low efficiency, which brings into question the homogeneity of the treated cell population and therefore the ability to completely kill the cells when GVHD signs appear. The cells must be allowed to proliferate for a period of weeks, which means that there is a delay between the time the cells are harvested and the transfected cells are ready for infusion. Finally, and most significantly, the cells transfused into humans will contain modified genetic material, which might prove to have a deleterious effect on the host in the long run.

Ionizing radiation ( $\gamma$ -radiation or X-radiation), or UV light (UVC  $\lambda$ =200-280 nm; UVB  $\lambda$ =280-320 nm; UVA  $\lambda$ =320-400 nm) have been used to treat blood products to induce DNA damage in cells. The currently accepted method for transfusion associated GVHD prevention is  $\gamma$ -radiation treatment (2500 cGy). The clinical dose of  $\gamma$ -irradiation (2500cGy) used for prevention of TA-GVHD results in a  $10^5$  to  $10^6$  fold reduction in viable T-cells. However,  $\gamma$ - and ionizing radiation in general, are not specific for nucleic acids. As a result of their high energy,  $\gamma$ -radiation and UVC also attack proteins and other cellular components, causing significant non-specific damage (Deeg, H.J. *et al.*, Blood Cells 18:151-162, 1992). To prevent GVHD, UVB has been used to abolish rat lymphocyte proliferation while preserving BM progenitor cell and primitive hematopoietic stem cell viability for engraftment. At a UVB treatment dose of 4,000 J/cm<sup>2</sup>, CTL activity was reduced but was still present (Gowing, H. *et al.*, Blood 87:1635-1643, 1996). However, UVB irradiation also leads to significant cell damage, especially if lamps with a broad emission spectrum are used (Gowing, H. *et al.*, Blood 87:1635-1643, 1996; Pamphilon, A.A. *et al.*, Blood 77:2072-2078, 1991). Surface molecule chemical modification and membrane rupture leading to lower cell viability and induction of immune responses have been reported, probably due to the ability of UVB to induce chemical changes on proteins and lipids, especially on unsaturated bonds. These

limitations are due to the fact that UVB is not selective for nucleic acids only, but modifies any chemical group which absorbs between 280 and 320 nm.

UVA irradiation in the presence of the photosensitizer 8-methoxy psoralen (8-MOP), used in the treatment of human leukocytes was shown to inhibit both the stimulating ability and proliferation of peripheral blood leukocytes, in mixed lymphocyte culture reaction *in vitro* (Kraemer, K.H. *et al.*, J. Inv. Derm. 77:235–239, 1981; Kraemer, K.H. *et al.*, J. Inv. Derm. 76:80–87, 1981). However, UVA plus psoralen treatment, as described in these reports, has resulted in the inhibition of surface antigen expression, cytokine synthesis (IL-1, IL-6, IL-8 and TNF) and transcription of cytokine mRNA (Gruner, S. *et al.*, Tiss. Ant. 27:147–154, 1986; Neuner, P. *et al.*, Photochem. Photobiol. 59:182–188, 1994). Therefore, the photochemical treatment conditions described in these prior studies, while inhibiting proliferation and reducing nonspecific cell damage, also inactivated T cell immune function; these treatment conditions would not be likely to produce cells effective to provide any immune protection or to induce a GVL effect, for transfusion purposes. Finally, UVA plus 8-MOP treatment of spleen and marrow cells has been used successfully for the reduction of GVHD in a murine model (Ullrich, S.E. J. Inv. Derm. 96:303–308, 1991). As with all the previous reports, this study addressed only the GVHD aspect of the donor leukocytes and did not provide leukocytes with immune protective or GVL function.

Clearly, the need exists for new approaches to suppress the induction of GVHD across allogeneic histocompatibility barriers, that do not require the systemic exposure to pharmaceuticals. Such a method should provide some immune protection to immunocompromised individuals and/or allow for killing of residual cancer, but should prevent proliferation of the infused cells in the host.

The invention described and claimed herein addresses and overcomes these and other problems associated with the prior art by providing methods of preparing leukocytes that do not induce GVHD yet maintain leukocyte function. This and other advantages provided by the methods of the present invention will be apparent from the detailed description below.

#### SUMMARY OF THE INVENTION

The present invention provides an effective method for treating leukocytes from an allogeneic donor to produce leukocytes that are unable to proliferate but maintain viability

and immune function effective to promote destruction of a diseased cell or pathogen. Since the treated leukocytes are incapable of proliferation, they are unable to induce GVHD when introduced into an allogeneic host (in a syngeneic or autologous transfusion, GVHD is not an issue) and are therefore ideal for use in DLI for various clinical indications and especially for the treatment of cancer and to provide immune function to an immunocompromised mammal.

Various aspects of the present invention include the following:

An isolated cell population comprising a population of leukocytes wherein a portion of the leukocyte population is non-proliferating, such that the cell population is incapable of eliciting graft-versus-host disease (GVHD) in an allogeneic host; and a portion of the leukocyte population retains immunological activity, including the ability to promote destruction of a diseased cell or a pathogen. Preferably, at least 90% of the leukocytes in the population are non-proliferating.

The population of leukocytes of the preceding embodiments is further characterized as 1) preferably comprising T cells, NK cells and antigen presenting cells; 2) capable, upon appropriate stimulation, of synthesizing and/or secreting cytokines such as IL-2, IFN- $\gamma$ , IL-10 and GM-CSF; 3) capable of expressing surface markers characteristic of T-cell activation and immune function, such as CD4, CD8, CD16 and CD56, among others; and 4) exhibiting an increased ratio of killer function to proliferative function, compared to untreated leukocyte populations.

The leukocyte population of the invention is, on the whole, incapable of proliferation but retains immunological activity. This leukocyte population will be capable of the following activities, among others:

1. Promoting destruction of a diseased cell, an infected cell, or a pathogen.
2. Facilitating the engraftment of a second population of cells. The second population of cells can be a suspension of cells or an organized collection of cells, such as a patch of tissue or an organ. Such cells can include, for instance, hematopoietic cells, myeloid cells, leukocytes, bone marrow cells, islet cells, hepatic cells, neuronal cells, myocardial cells, mesenchymal cells and endothelial cells.
3. Promoting immune reconstitution.
4. Immunotherapy.
5. Treatment of mixed chimerism.

6. Promoting a graft-vs.-leukemia (GVL) response.

In one embodiment, the population of leukocytes is effective to promote destruction of a cancerous cell. Preferably, the cancerous cell is selected from the group consisting of Chronic Myelogenous Leukemia (CML) cell, Chronic myelomonocytic Leukemia (CmML) cell, Chronic Lymphocytic Leukemia (CLL) cell, Acute Myelogenous Leukemia (AML) cell, Acute Lymphoblastic Leukemia (ALL) cell, multiple myeloma (MM) cell, Hodgkin's lymphoma cell and non-Hodgkin's lymphoma cell. In preferred embodiments, the cancerous cell is a Chronic Myelogenous Leukemia (CML) cell or a multiple myeloma cell.

In another embodiment, the population of leukocytes is effective to promote destruction of a diseased cell which is an infected cell. Infected cells include cells infected with a virus. Preferably, the virus infecting the cell is selected from the group consisting of cytomegalovirus (CMV), Epstein Barr virus (EBV), Adenovirus (Ad) and Kaposi's Sarcoma associated Herpes virus.

In yet a third embodiment, the leukocyte population is effective to promote destruction of a pathogen. Pathogens include bacteria, fungi and parasites.

In still another embodiment, the leukocyte population is effective to facilitate engraftment by a second population of cells. The cells used for engraftment can be, for example, hematopoietic cells, myeloid cells, leukocytes, bone marrow cells, islet cells, hepatic cells, neuronal cells, myocardial cells, mesenchymal cells and endothelial cells. In addition, the second population of engrafted cells can comprise a solid organ or portion thereof.

Subsets of the leukocyte population described above, selected by various methods, are also provided by the invention. The subsets include lymphocytes and T-lymphocytes; and subsets can be obtained by both positive and negative selection based on, for example, surface marker expression. Preferred surface markers include CD8, CD4, CD16 and CD56. Methods for selection of subsets of leukocyte populations from whole blood include leukophoresis and red cell removal.

In another embodiment, the invention provides a variety of therapeutic methods utilizing cell populations as described above, including donor leukocyte infusion, leukocyte add-back, immune reconstitution, adoptive immunotherapy, treatment of mixed chimerism, and methods for enhancing engraftment of a second population of transplanted cells. For use in methods for enhancing engraftment of a second population of

transplanted cells, the cell populations of the invention can be introduced into the host prior to, at the same time as, or subsequent to transplantation of the second population of cells.

Also provided by the invention is a method for preparing a treated leukocyte population wherein the leukocyte population as a whole is non-proliferating and incapable of eliciting graft-versus-host disease (GVHD) in an allogeneic host, the method comprising the steps of:

i) providing a sample comprising a population of leukocytes; and  
ii) combining the sample with a compound capable of forming a covalent bond with a nucleic acid, in an amount such that the compound forms about 1 to  $10^4$  adducts per  $10^8$  base pairs of genomic DNA of the leukocytes, thereby inhibiting proliferation but maintaining immunological activity, including the ability of the leukocyte population to promote destruction of a diseased cell or a pathogen.

In a preferred embodiment, the compound is present in an amount effective to form from about 5 to  $10^3$  adducts per  $10^8$  base pairs of genomic DNA of the leukocytes. Preferably, the method results in proliferation being inhibited in at least 90% of the T cells within the treated leukocyte population.

Preferably, the compound capable of forming a covalent bond with a nucleic acid further comprises a nucleic acid binding moiety capable of binding non-covalently with a nucleic acid.

In one embodiment, the compound comprises: a nucleic acid-binding moiety; a moiety capable of reacting to form a covalent bond with nucleic acid; and a frangible linker covalently linking the nucleic acid-binding moiety and the effector moiety.

Preferably, the nucleic acid-binding moiety is an aromatic intercalator and the effector moiety is a mustard.

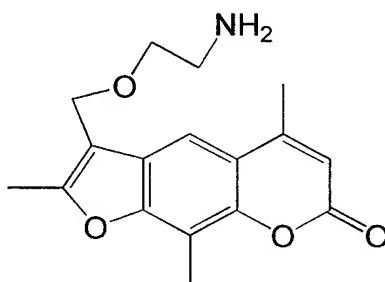
In a preferred embodiment of the method for preparing a treated leukocyte population, the compound capable of forming a covalent bond with a nucleic acid comprises a photoactivatable moiety, which upon electromagnetic stimulation, forms a covalent bond with a nucleic acid. When using a compound having a photoactivatable moiety i.e., a photoactivatable compound, the method will further comprise the step of exposing the sample of leukocytes that have been combined with the compound to light, to

photoactivate the photoactivatable moiety, thereby resulting in the photoactivatable moiety forming a covalent bond with leukocyte genomic DNA.

In one embodiment of the preceding method, the compound having a photoactivatable moiety is selected from the group consisting of furocoumarins, actinomycins, anthracyclines, anthramycins, benzodipyrone, fluorenes, fluorenones, monostrals blue, norphillin A, organic dyes; phenanthridines, phenazathionium salts, phenazines, phenothiazines, phenylazides, quinolines and thioxanthones acridines and ellipticenes. A preferred furocoumarin is a psoralen. Preferred psoralens include PAP, 8-methoxy psoralen (8-MOP), 4'-aminomethyl 4, 5', 8-trimethylpsoralen (AMT), 5-methoxy psoralen, and trioxalen 4, 5' 8-trimethylpsoralen.

Where the compound used to treat the leukocytes is psoralen, the psoralen is preferably present at a concentration in the range of  $10^{-4}$  to  $150 \mu\text{M}$  and the sample of leukocytes is exposed to ultraviolet light having a wavelength in the range of 200 to 450 nm, preferably between 320 and 400 nm. Preferably, the ultraviolet light is provided at a dosage of between  $10^{-3}$  to  $100 \text{ J/cm}^2$ . The sample of leukocytes will be exposed to the ultraviolet light for a period of 1 second to 60 minutes.

In a preferred embodiment, the method for preparing a treated leukocyte population according to the above embodiments, employs the psoralen referred to herein as S-59, having the formula:



and salts thereof. S-59 will be used at a concentration in the range of  $10^{-4}$  to  $150 \mu\text{M}$ , even more preferably, in the range of  $10^{-3}$  to  $150 \mu\text{M}$ . The sample of leukocytes combined with S-59 is exposed to ultraviolet light having a wavelength in the range of 200 to 450 nm, preferably between 320 and 400 nm. The sample of leukocytes combined with S-59 is preferably exposed to the ultraviolet light at a dosage of between  $10^{-3}$  to  $100 \text{ J/cm}^2$ , more preferably,  $3 \text{ J/cm}^2$ . To photoactivate the S-59, the sample of leukocytes is preferably exposed to the ultraviolet light for a period of between 1 second to 60 minutes, more preferably, for 1 minute. The sample of leukocytes will preferably be provided at a



cell density of  $10$  to  $10^9$  cells per mL, more preferably between  $10^2$  and  $10^8$  cells per mL, most preferably at  $2 \times 10^6$  cells per mL.

A leukocyte population produced according to the preceding methods, is provided, including a leukocyte population specifically treated with S-59.

5 Yet another aspect of the invention is a method of promoting destruction of a diseased cell or a pathogen, comprising mixing a leukocyte population produced by the aforementioned methods with a population of allogeneic cells containing the diseased cell or pathogen. The method can be performed *in vitro* or *in vivo*. In one preferred embodiment, the leukocyte population is mixed with the population of allogeneic cells of a  
10 mammalian host *in vivo* by donor leukocyte infusion into said host. Preferably, the donor leukocyte infusion is administered to a mammalian host suffering from relapse from leukemia or multiple myeloma post BMT.

In a preferred embodiment of the method of promoting destruction of a diseased cell or a pathogen, the diseased cell is a cancerous cell. Cancerous cells from the  
15 following cancers are encompassed: Chronic Myelogenous Leukemia (CML) cell, Chronic myelomonocytic Leukemia (CmML) cell, Chronic Lymphocytic Leukemia (CLL) cell, Acute Myelogenous Leukemia (AML) cell, Acute Lymphoblastic Leukemia (ALL) cell, multiple myeloma (MM) cell, Hodgkin's lymphoma cell and non-Hodgkin's lymphoma cell. In a most preferred embodiment, the cancerous cell is a Chronic  
20 Myelogenous Leukemia cell or a multiple myeloma cell.

Another type of preferred cancerous cell is a cancerous cell selected from the group consisting of breast cancerous cell, lung cancerous cell, ovarian cancerous cell, testicular cancerous cell, prostate cancerous cell, colon cancerous cell, melanoma cell, renal carcinoma cell, neuroblastoma cell, head tumor cell and neck tumor cell.

25 In another preferred embodiment of the method of promoting destruction of a diseased cell or a pathogen, the diseased cell is an infected cell. Preferred infected cells encompass cells infected with a virus such as cytomegalovirus (CMV), Epstein Barr virus (EBV), Adenovirus (Ad) or Kaposi's Sarcoma associated Herpes virus.

In yet another embodiment of the preceding method of promoting destruction of a  
30 diseased cell or a pathogen, the leukocyte population has been stimulated with one or more epitopes of an antigen specific to the diseased cell or pathogen, to expand the number of cytotoxic T cells specific to the antigen. Stimulation or antigen-specific T cell expansion can be performed *in vivo*, *ex vivo*, or *in vitro*. *In vivo* stimulation is performed by

vaccination of the leukocyte donor with an antigen specific to the diseased cell or pathogen prior to isolation of the leukocyte population from the donor. In the case where the disease is CML, the leukocyte population is preferably stimulated with a *bcr-abl* antigen of the CML cell. In the case of multiple myeloma, the leukocyte donor can be vaccinated with the idiotype antigen of the patient's myeloma cell.

In still another embodiment of the preceding method of promoting destruction of a diseased cell or a pathogen, the leukocyte population has been stimulated with a mitogen. Stimulation is preferably conducted *in vitro* with a mitogenic composition such as a phorbol myristate acetate (PMA) plus ionomycin, or a phytohemagglutinin.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** shows the reduction of proliferating T-cells after PCT with various concentrations of S-59 (squares), AMT (triangles) and 8-MOP (circles) (UVA = 1J/cm<sup>2</sup>) (see Example 1).

**Figure 2** shows <sup>3</sup>H-thymidine incorporation by effector cells treated with S-59 at the various drug dosages, in a MLR assay (see Example 2).

**Figure 3** shows the level of IL-2 production in a MLR by effector cells photochemically treated with S-59 at the various drug dosages (see Example 2).

**Figure 4** shows the level of IFN-γ production in a MLR by effector cells photochemically treated with S-59 at the various drug dosages (see Example 2).

**Figure 5** shows the levels of IL-8 generated after PCT of leukocytes in PC with different concentrations of S-59 and 0.5 J/cm<sup>2</sup> UVA, as a function of time after PCT (see Example 2)

**Figure 6** shows the levels of CD69 marker expression after PCT with different doses of AMT and 1 J/cm<sup>2</sup> UVA, as a function of time after treatment (see Example 3)

**Figure 7** shows psoralen-DNA adduct formation following photochemical treatment with various psoralens in PC. S-59 (squares); AMT (triangle); 8-MOP (circles); 1.9 J/cm<sup>2</sup> UVA (see Example 3).

**Figure 8** shows the effects of S-59 + UVA treatment on proliferation of treated cells, measured by  $^3\text{H}$  thymidine incorporation in a MLR assay. NR denotes untreated cells; UV denotes cells irradiated with UVA in the absence of S-59. Also shown are the results obtained when cells were irradiated with  $3 \text{ J/cm}^2$  UVA in the presence of three different concentrations of S-59. See Example 14 for details.

**Figure 9** shows the effects of S-59 + UVA treatment on proliferation of treated cells, measured after activation of treated cells with anti-CD3 antibody. See Example 14 for details.

**Figure 10A** shows production of IL-2 by human PBMC that have been photochemically treated with S-59 + UVA and stimulated in a MLR. Measurements are by sandwich ELISA. See Example 14 for details.

**Figure 10B** shows production of IFN- $\gamma$  by human PBMC that have been photochemically treated with S-59 + UVA and stimulated in a MLR. Measurements are by sandwich ELISA.

**Figure 11A** shows CD69 expression in treated and control cells measured at different times (hours) after activation by anti-CD3.

**Figure 11B** shows CD25 expression in treated and control cells measured at different times (hours) after activation by anti-CD3.

**Figure 12** shows CD40L expression in treated and control untreated cells measured at different times (hours) after activation by anti-CD3.

**Figure 13** shows cytotoxic T-cell activity of photochemically-treated, activated leukocytes, measured by  $^{51}\text{Cr}$  release from target cells that were co-incubated with the treated leukocytes. E:T represents effector cell to target cell ratio.

**Figure 14** shows measurements of average body weight in irradiated mice which received a MHC-mismatched bone marrow transplant along with S-59 + UVA-treated splenocytes, and were then challenged with leukemia cells three days after transplant. Splenocytes were exposed to UVA in the presence of  $0.01 \mu\text{M}$  S-59 for different amounts of time, as indicated in the figure.

**Figure 15** shows analysis of GVL activity in leukemia-challenged mice, which received bone marrow transplants with or without infusion of treated leukocytes. GVL activity is demonstrated by percent leukemia-free survival (survival ratios given in parentheses)..

**Figure 16** shows analysis of proliferative ability of S-303-treated responder cells, measured by  $^3\text{H}$ -thymidine incorporation between 6 and 7 days after co-culture with gamma-inactivated allogeneic stimulator cells in a MLR.

**Figure 17** shows levels of IFN- $\gamma$  in MLR supernatants in which the responder cells were treated with different concentrations of S-303 prior to co-culture.

#### DETAILED DESCRIPTION

##### Abbreviations

The following abbreviations are used:

BMT: bone marrow transplantation

DLI: donor leukocyte infusion

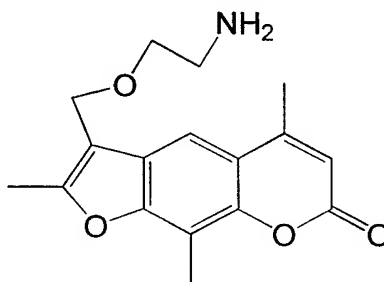
LDA: limiting dilution analysis

MLR: mixed lymphocyte reaction

PA: photochemically arrested; PCT: photochemical treatment; PI-DLI: Photochemically inactivated donor leukocyte infusions; PUVA: Psoralen and ultraviolet A radiation.

PAP: 4'- and 5'-primary amino-substituted psoralen

S-59: a primary amino-substituted psoralen having the following formula:



PBSC: peripheral blood stem cell

PC: platelet concentrate

TA-GVHD: transfusion associated graft-versus-host -disease

PHA: phytohemagglutinin

### Definitions

“Allogeneic” refers to the relationship that exists between genetically nonidentical members of the same species, i.e., the members are not syngeneic.

“Host” is used interchangeably with “recipient” and refers to a mammalian recipient of allogeneic donor leukocytes. Generally, the host will be a human but other mammals such as mice, dogs, cats, monkeys, horses, etc. are encompassed.

A “leukocyte” refers to any white blood cell including the lineage progenitor cells. “Antigen presenting cells” include macrophages, and dendritic cells which are present in low numbers in peripheral blood, as well as their respective precursor cells. Leukocytes are present in circulating blood and in bone marrow as well as in the myelopoietic, lymphoid and reticular sites of the reticuloendothelial system.

A “leukocyte population” as used herein refers to a population of leukocytes that contains more than one leukocyte cell type and will include at least T cells, antigen presenting cells and NK cells.

“Donor leukocytes” refer to leukocytes that are not endogenous to the host animal but are derived from an allogeneic donor. For in vitro and non-human applications, the leukocyte population and donor leukocytes can be from a mammal including rodents, rabbits, dogs, etc.

By “purified” is meant that the leukocyte population has been isolated from the body and processed to be substantially free from non-leukocyte cell types and preferably from other contaminants such as cellular debris that are present in the source of the leukocytes, the source generally being peripheral blood, bone marrow or even splenocytes. Preferably, the leukocyte population is purified such that it contains less than 13% red blood cells (RBC), more preferably less than 5% , even more preferably, less than 1%. In a most preferred embodiment, the hematocrit level is less than 0.5%. Methods for purification of leukocytes are described below.

“Treated leukocytes” refers to leukocytes that have been exposed to or contacted with a compound capable of forming one or more covalent bonds with nucleic acid. The leukocytes can be “treated” by PCT or with an alkylator compound. “Photochemically inactivated” or “photochemically arrested” (PA) or “PCT-arrested” leukocytes refers to

PCT leukocytes that are incapable of proliferation even upon stimulation, and does not imply that the leukocytes are inactivated in protein expression and immune function.

A population of leukocytes of the invention contains but is not limited to cells that are “non-proliferating”. Cells are “non-proliferating” if they are arrested in DNA replication and are thus, unable to divide to generate new cells even upon stimulation with reagents such as mitogens, cytokines, antigens, antibodies or other proliferation stimuli. It is not essential that the methods of the present invention result in replication arrest of every leukocyte in the preparation. For the purpose of this invention, it is adequate that the leukocyte population is arrested in proliferation in at least 90%, more preferably in at least 95% of the leukocytes within the purified population, provided that the small fraction of leukocytes capable of proliferation is insufficient to cause GVHD in an allogeneic host. In a most preferred embodiment, 99% or more of the leukocytes within the population are non-proliferating. Proliferation activity can be assayed, e.g., by limiting dilution assay (LDA), or by <sup>3</sup>H-thymidine incorporation separately, or as part of a MLR assay as described in the Examples below. In the most preferred embodiment, T-cells in the population are proliferation inhibited to the limit of detection by LDA. The results of these assays have been shown to be predictive of the ability of the treated leukocytes to proliferate in vivo.

“Graft-versus-host disease” or “GVHD” results from clonal expansion of donor T-cells present in the transfused BMT or DLI, proliferating and colonizing host tissues. The occurrence and severity of GVHD has been shown to be associated with the presence of clonable T-cells, (see Kernan, N.A. *et al.*, Blood 68:770–773, 1986). In patients who are HLA-matched with their donors, the occurrence of GVHD is attributed to minor histocompatibility antigens. GVHD onset is due to the absence of immune function in immunosuppressed patients, a conditioning necessary for the BMT to be successful. The donor T-cells are able to proliferate unchallenged in that environment and attack host tissues, causing the pathological symptoms. At the cellular level, antigen-presenting-cells of the host interact with the T-cells of the donor in the context of the major histocompatibility complexes (MHC) I and II and induce their activation against cells bearing host-specific (minor) antigens. This leads to clonal proliferation of the activated T-cells, which attack the host tissues and release cytokines. Cytokines secreted by the T-cells also activate a variety of other effector cells of the host which adds to the tissue damage through the additional generation of cytokines (cytokine storm). See e.g.,

Burakoff, S.J. *et al.*, Graft-Versus-Host Disease Immunology, Pathophysiology, and Treatment, in Brinkhous KMS, S.A. (ed): Hematology, vol. 12 (ed 1st). New York, Marcell Dekker, Inc., 1990, p 725. Burakoff, S.J. *et al.*, Graft-Versus-Host Disease Immunology, Pathophysiology, and Treatment, in Brinkhous KMS, S.A. (ed): Hematology, vol. 12 (ed 1st). New York, Marcell Dekker, Inc., 1990, p. 725.

When GVHD is diagnosed, the patient is usually given a high dose of immunosuppressive drugs to suppress the GVHD. However, these drugs also render the graft leukocytes ineffective in GVL and the patient may relapse into leukemia.

The capability of a leukocyte population to elicit GVHD can be determined in vivo, e.g., as described in Example 4 below, or in vitro by limiting dilution assay (LDA), or by MLR, as described in Example 5 below. The leukocyte population is judged incapable of eliciting GVHD if the number of clonable T cells in the population, as assayed by LDA, is within about  $10^{-3}$  to  $10^{-4}$  of that present in the untreated leukocyte control population (positive control for GVHD and proliferation). In vivo, lack of clinical symptoms of GVHD (symptoms described in Example 4) in an allogeneic recipient of the leukocyte population indicates incapability of the population to elicit GVHD

A leukocyte population is considered "effective to promote destruction of a diseased cell or pathogen" if it includes leukocytes that are able to participate, directly or indirectly, in immune responses that are effective to kill or clear the targeted diseased cell or pathogen from the body, or to limit the proliferation of the diseased cell or pathogen. Not every leukocyte present in the leukocyte population has to be able to promote destruction but the population as a whole should be effective in that regard.

It should be recognized that the diseased cell or pathogen can be destroyed via any mechanism. It is not the intention of the invention to have the methods of treating leukocytes be limited by particular mechanisms by which the treated leukocytes can mediate destruction. The diseased cell may be destroyed by cytolysis by T or NK cells, but can also be killed by other mechanisms such as by being induced to undergo apoptosis. The diseased cell or pathogen can also be destroyed by antibody-dependent cell-mediated cytotoxicity (ADCC), by phagocytosis by macrophages, or be cleared by the reticuloendothelial system via any immune mechanism naturally existing in a mammal. A pathogen that resides inside a host cell can be destroyed indirectly by killing the host cell upon which it depends for its survival. Mechanisms by which the immune system rids the body of malignant cells, infected cells or pathogens are discussed in standard immunology

textbooks, e.g., in Basic and Clinical Immunology, 8th edition, Daniel P. Stites, Abba I. Terr and Tristram G. Parslow (eds.), Appleton & Lange, Norwalk, CT, 1994. The “effective” treated donor leukocyte can be an effector cell such as a cytotoxic T cell (CTL), a NK cell, or a macrophage that directly kills a target diseased cell or pathogen, or that induces the diseased cell to undergo apoptosis. Alternatively, the treated donor leukocyte can mediate destruction by stimulating other leukocytes, e.g., the leukocytes from a transplanted bone marrow, a previous DLI, or the host’s own leukocytes, to perform the killing or clearance. Stimulation of other leukocytes can be by surface antigen expression, cytokine secretion or any appropriate mechanism. Cytolysis can occur via MHC-restricted and/or non-MHC restricted (such as by NK cells) mechanisms.

The effectiveness of a leukocyte population to promote destruction of a diseased cell or pathogen can be measured by various assays, such as by MLR or by  $^{51}\text{Cr}$  release assay as described below in the Examples. Cytolytic effectiveness is demonstrated, e.g., by the ability to mediate killing of a leukemic cell in a  $^{51}\text{Cr}$  release assay. For the purposes of this invention, a leukocyte population is considered effective to promote destruction of a diseased cell if, in an appropriate assay (e.g., the  $^{51}\text{Cr}$  release assay), the population exhibits cytolytic activity at a level of at least about 20% above that of the negative control population. The types of diseased cells and pathogens targeted for killing are provided below.

Generally, effectiveness to promote destruction will require that the population of leukocytes maintains a level of protein synthesis to allow production and secretion of proteins including signal transduction molecules such as cytokines. Preferably, there is also expression of surface antigens including receptors, adhesion molecules and co-stimulatory molecules on the plasma membrane. Cell viability and membrane integrity are critical for mediating GVL effect. “Cell viability” is defined herein as survival in circulation i.e., in the blood stream and other tissues.

While some cytokine production may be inhibited, preferably cytokine production is maintained at least 70%, more preferably at 80%, even more preferably greater than 90%, and most preferably greater than 95%, of the level before treatment with the aforementioned compound. Preferably, the leukocyte population includes leukocytes able to secrete at least interleukin-2 (IL-2) and interferon- $\gamma$  (IFN- $\gamma$ ) under stimulation. These cytokines are relevant because of their established role in T-cell activation and GVHD/GVL. Other relevant cytokines include granulocyte-macrophage colony-



stimulating factor (GM-CSF) and interleukin-10 (IL-10). Secretion of cytokines can be readily assayed e.g., by using cytokine assay kits (e.g. kits from R&D Systems) as described in the Examples section below.

5 The surface antigens (also referred to as surface markers) expressed on the leukocytes in the purified leukocyte population will depend on the specific cell type. Preferably, the population will include leukocytes expressing the following surface antigens which are known to be involved in interactions associated with T-cell and NK cell activation and immune function: CD2, CD28, CTLA4, CD40 ligand (gp39), CD18, CD25, CD69 (lymphocyte activation marker) and CD16/CD56, antigens. Preferably, the  
10 leukocytes will also express other surface markers including MHC Class I and Class II, CD8, CD4, CD3/TcR (T cell receptor), adhesion molecules such as CD54 (ICAM -1), LFA-1 and VLA-4, and other co-stimulatory molecules. The expression of surface antigen can be detected and measured by various assays such as by staining cells with antibodies specific to the particular antigen and detecting the antibodies which have been labeled  
15 directly or indirectly, by standard FACS-SCAN analysis. Other methods known in the art include immunoprecipitation of surface antigens and immunoblotting.

“Diseased cell” as used herein, refers to a cancerous cell, an infected cell, or a cell in any type of pathological state, which may be present in vivo or in vitro. The cancerous or malignant cell can be from any type of cancer, of any tissue or cell type origin. Such  
20 malignant or cancerous cells include but are not limited to cells of the following malignancies: Leukemia including Chronic Myelogenous Leukemia (CML), Chronic Lymphocytic leukemia (CLL), Acute Myelogenous Leukemia (AML), and Acute Lymphoblastic Leukemia (ALL); Multiple myeloma (MM); Non-Hodgkin lymphoma and Hodgkin’s disease (lymphoma); solid tumors, including breast, lung, ovarian and testicular  
25 cancers, prostate cancer, colon cancer, melanoma, renal carcinoma cell, neuroblastoma, and head and neck tumors. Infected cell encompasses cells infected by any of the following: a virus, a bacterium, a fungus, a parasite or any other pathogenic microorganism.

“Pathogen” is defined as any agent containing nucleic acid and capable of causing  
30 disease in a human, other mammals, or vertebrates. Examples of pathogens are bacteria, viruses, protozoa, fungi, yeasts, molds, and mycoplasmas which cause disease in humans, other mammals, or vertebrates. The genetic material of the pathogen may be DNA or RNA, and the genetic material may be present as single-stranded or double-stranded

nucleic acid. The pathogen can be outside a cell or residing inside a cell as exemplified by HIV in a macrophage.

The terms donor leukocyte “infusion” and “transfusion” are used interchangeably herein and refer to the same procedure.

5 “Light dose or dosage” or “PCT dose”, as measured in units of  $\text{J}/\text{cm}^2$  refers to the total light energy per unit area that a sample of leukocytes receives during PCT. Light dosage can be altered by varying the intensity of light and time of exposure of the cell sample to the light.

10 “Intensity” of light as measured in units of  $\text{mW}/\text{cm}^2$  refers to the energy of light received per unit of the sample, per second.

A “photoactivatable moiety” is defined herein as a moiety which undergoes a chemical change in response to electromagnetic radiation. When the compound including the photoactivatable moiety is photoactivated by electromagnetic radiation, the photoactivated moiety forms a covalent bond with a nucleic acid to form a compound:  
15 nucleic acid complex, referred to herein as an “adduct”.

“Aromatic intercalator” refers to a compound or region thereof having aromatic ring structure, that can intercalate nucleic acids. Aromatic intercalators include but are not limited to, anthracene, acridine, naphthalene, naphthoic acid, etc.

20 An “isolated population of cells,” as used herein, comprises a population of cells existing outside of the organism in which the cells normally reside.

“Immunological activity” as used herein, refers to functions expressed by cells of the immune system, such as T-cells, B-cells, NK cells, antigen-presenting cells (APCs) and others, such functions including, but not limited to, synthesis and secretion of cytokines, cell-mediated cytotoxicity, synthesis and secretion of antibodies, processing and  
25 presentation of antigens and antigen fragments, and expression of surface markers characteristic of immune cells.

#### Incorporation by Reference

30 References cited within this application, including patents, published patent applications and other publications, are hereby incorporated by reference.

### Description of Preferred Embodiments

Various preferred aspects of the present invention are summarized below and further described and illustrated in the subsequent detailed descriptions and examples.

5           The present invention provides a method of treating a population of isolated leukocytes so as to render a portion of the population non-proliferating to the extent that the population causes minimal GVHD while still retaining sufficient immune function effective to promote destruction of a diseased cell or pathogen. By “minimal GVHD” is meant that the extent of the GVHD is insufficient to result in mortality of the mammal or  
10           to abolish the ability of the leukocyte population to promote destruction of a diseased cell or pathogen and/or to mediate GVL effect.

          GVHD is clearly associated with the clonal expansion of the donor T-cells and, in principle, any treatment which removes, inactivates or kills leukocytes would be effective against it. GVL, on the other hand, is associated with an immune response of the donor  
15           leukocytes and may be the result of cytolytic action, presentation of antigens, synthesis of specific cytokines or a combination of the above. This necessitates some of the functions of the donor leukocytes to be intact for a GVL effect to be observed. The inactivation of donor leukocyte function in efforts to avoid GVHD also renders the donor BM or  
20           leukocytes ineffective in providing necessary immune functions to an immunocompromised or BMT host.

          In order for a treatment to be able to eliminate GVHD but still provide immune protection and/or induce a GVL effect in an allogeneic host, the proliferating ability of leukocytes must be selectively removed, while retaining some level of leukocyte function. The invention provides populations of leukocytes with these characteristics.

25           The isolated population of leukocytes of the present invention provide various advantages over those previously described and used. One significant benefit is that eliminating the onset of GVHD will allow the safe infusion of donor leukocytes into patients. As a result, the host, who is lacking an immune system or has a chimeric immune system, can receive either a larger dose of treated donor leukocytes and/or multiple DLIs,  
30           thus enhancing the efficacy of treatment of the disease. Previously, the threat of GVHD limited the number of leukocytes infused.

          The population of non-proliferating leukocytes is effective to provide the host with sufficient immune defense to combat cancer and infections. Such leukocytes are useful for

treating relapsing hematological malignancies such as chronic myelogenous, acute myelogenous, acute lympholytic, multiple myeloma and other forms of leukemia and myeloma, as part of post-BMT treatment or as an alternative to BMT. A GVL effect from the DLI is useful not only to remove minimal residual disease but can also be applied to the treatment of a higher malignant cell load, especially since absence of leukocyte expansion allows for the safe infusion of greater numbers of cells. The treated leukocyte populations of the invention are also useful for the treatment of certain solid tumors (*e.g.*, breast cancer and renal cell carcinoma) that are susceptible to immunomodulatory therapy and in preventing graft rejection in both matched and mismatched allo BMT procedures. Various other clinical indications for which the present treated leukocytes can be applied in treatment are described in detail below.

The method of treating leukocytes of the invention involves the following. Leukocytes can be obtained from bone marrow, cord blood or whole blood. The most convenient source of leukocytes is peripheral blood. Methods of isolating leukocytes from other sources are described in the scientific literature. Whole blood is processed to purify and enrich for leukocytes by, for example, red cell removal or leukaphoresis. The resultant purified population of leukocytes is about 99% free of red blood cells which are of different size and density compared to leukocytes and are readily removed by these processes.

The population of leukocytes is then mixed with a compound, and treated under conditions that are effective to arrest proliferation of the leukocytes without compromising cell viability and integrity. Unreacted compound can be removed, or the compound may become inactive with time, so that removal is not necessary. In a preferred embodiment, "compound" will refer to a compound capable of forming a covalent bond with a nucleic acid. Covalent bond formation may form about from 1 to  $10^4$  adducts, preferably from 5 to  $10^3$  adducts, most preferably  $10^3$  adducts per  $10^8$  base pairs of leukocyte genomic DNA. It is important that the treatment conditions minimize nonspecific damage to proteins and other cellular components and avoid membrane damage. Membrane integrity of the majority of treated leukocytes is necessary for a GVL effect to take place. These treatment conditions will also be effective to render the treated leukocytes incapable of eliciting GVHD if introduced into an allogeneic host, or non-responsive in an MLR assay in vitro. In addition, the treated leukocytes should maintain protein expression effective to

accomplish immune function, specifically to promote destruction of a diseased cell or pathogen either in vivo or in vitro.

Properties of suitable compounds capable of forming a covalent bond with a nucleic acid, preferably double stranded DNA, are disclosed below. The compounds may include a moiety capable of forming a covalent bond with a nucleic acid which is photoactivatable or chemically reactive. The compound inhibits proliferation of cells by forming covalent bonds with the genomic DNA, thus interfering with the function of DNA polymerases and rendering the leukocytes non-proliferating.

The number of covalent adducts that the compound forms with leukocyte nucleic acid can be modulated so that the compound inhibits proliferation but maintains immune functions effective to promote destruction of a diseased cell or pathogen. With alkylator compounds, the effect can be modulated by adjusting the concentration of the compound and the length of time the leukocytes are contacted with the compound before removing unbound compound. The concentration required will depend on the characteristics of the particular compound, such as its solubility in aqueous solution and the DNA binding constant. The compound will typically be used at concentrations effective to generate about 1 to  $10^4$  adducts of the covalent binding compound per  $10^8$  base pairs of leukocyte genomic DNA, preferably about 5 to  $10^3$  adducts, even more preferably, about  $10^2$  to  $10^3$  adducts. Ideally, the conditions for treating the leukocyte population will generate about  $10^3$  adducts per  $10^8$  base pairs of genomic DNA. The lowest concentration of compound effective to achieve the leukocyte compositions of the present invention is the preferred concentration.

With photoactivatable compounds, the PCT effect can be modulated by adjusting the concentration of the compound, the wavelength of light, and the length of time of exposure to that light. The conditions for PCT using psoralen and other photoactivatable compounds are described in detail below.

Treatment conditions will be targeted to generate about 1 to  $10^4$  adducts of the covalent binding compound, per  $10^8$  base pairs of leukocyte genomic DNA, preferably about 5 to  $10^4$  adducts, even more preferably, about  $10^2$  to  $10^3$  adducts. Ideally, the treatment conditions will generate about  $10^3$  adducts per  $10^8$  base pairs of genomic DNA. The number of compound-DNA adducts resulting from treatment can be measured e.g., by using radiolabeled DNA binding compound as described in the Examples below.

Another type of compound that can be used in the treatment of leukocytes is a small molecule that acts as an inhibitor of DNA replication. Such small molecule replication inhibitors can optionally comprise a linker (frangible or otherwise) and an effector, as described above. Any of the steps of DNA replication can serve as a target for inhibition, including formation of an origin recognition complex (ORC), recruitment of the ORC to the origin, initiation of replication, elongation, *etc.* In addition, inhibitors of enzymes that facilitate the process of replication, such as helicases and topoisomerases, are useful. Examples of topoisomerase inhibitors include, for instance, camptothecin and daunomycin.

To combine the sample of leukocytes with a compound, the compounds of the present invention may be introduced into a suspension of leukocytes (leukocyte sample) in several forms. The compounds may be introduced as an aqueous solution in water, saline, a synthetic media such as “Sterilyte™ 3.0”, or in the solution that the leukocytes are suspended in. Solutions (infusion grade and non-infusion grade) appropriate for resuspending the leukocytes are provided below. “Synthetic media” is herein defined as an aqueous synthetic blood or blood product storage media. The compounds can further be provided as dry formulations, with or without adjuvants. The cell suspension is then agitated to mix in the compound.

The leukocytes to be treated with compound are resuspended in physiologically balanced solution such as plasma, synthetic media or a combination thereof. The leukocytes can be provided at a volume of from 200 mL to 1 liter. The leukocytes for treatment will preferably be contained in a reaction vessel such as a blood bag. Blood bags are known in the art.

The effect of treatment with the compound on the viability and function of the leukocyte population can be monitored by in vitro as well as in vivo assays to determine the optimum treatment conditions that minimize proliferation and GVHD activity while maximizing cytotoxic function and/or the GVL effect. Optimum conditions will vary with the properties of the compound used and will be validated for the disease or pathogen. In the case of photoactivatable compounds, the most preferred PCT condition will comprise the lowest concentration of compound and the lowest light dosage found sufficient for providing a population of leukocytes that is arrested in proliferation but effective to promote destruction of a diseased cell or pathogen. The population of treated leukocytes will be characterized as follows.

5 The cell viability of the population of treated leukocytes will be assessed. Cell viability can be measured in vivo, for example, by PCR analysis to detect sequences specific to donor leukocytes, as described in the Examples section below. Preferably, the population of leukocytes will have a cell viability of at least 3 weeks. Fast clearance of leukocytes upon treatment may affect their ability to induce an antileukemic response. Membrane integrity of the treated leukocytes is also necessary for a GVL effect to take place. Membrane integrity can be determined by trypan blue or propidium iodide dye exclusion. The PCT procedure, if used, is optimized for the light dose and/or the concentration of the compound which will maximize the number of leukocytes with intact membranes.

10 Proliferation activity can be measured, e.g., by Limiting Dilution Assay (LDA) or Mixed Lymphocyte Reaction (MLR) Assay, as described in the Examples below. The activity of the leukocytes in a Mixed Lymphocyte Reaction (MLR) assay will be predictive of the ability of the treated leukocytes to mediate GVHD in vivo. Alternatively, GVHD can be measured by monitoring GVHD symptoms and/or morbidity and mortality induced by GVHD in MHC matched animals transfused with the treated leukocytes.

15 Leukocyte activity can be determined by monitoring cytokine synthesis, expression of surface antigenic markers and ability to lyse target cells. Expression of surface antigenic markers is determined through the use of the appropriate antigen-specific antibodies and standard FACS-SCAN analysis. The presence of antigenic markers, CD2, CD28, CTLA4, CD40 ligand (gp39), CD18, CD25, CD69 (lymphocyte activation marker) and CD16/CD56, which are known to be involved in interactions associated with T-cell and NK cell activation and immune function, will be determined as a function of concentration of the covalent bonding compound and, if used, the PCT light dose.

20 Stability of surface molecules under the PCT conditions is not expected to be influenced given the mild nature of PCT on proteins. Treatment conditions, for example, PCT, will be chosen such that they do not adversely affect the expression of the surface molecules or reduce cytokine production below desired levels (preferred levels disclosed above).

25 Cytolytic activity indicative of GVL activity can be measured, e.g., by lysis of human or mouse leukemia cell lines in a <sup>51</sup>Cr release assay. The anti-leukemia effect can be assayed in vitro as described in Choudhury et al. Blood 89:1133-1142, 1997, or in vivo as described in Johnson et al. Blood 85:3302-3312, 1995. Activity against solid tumors can be tested, e.g., using murine models of solid tumors. The ability to mediate lysis of an

infected cell or a pathogen can be assayed using infected animals in an animal model. GVHD and GVL capabilities can also be measured in vivo as described in Examples 4 and 5 below.

All the above assays are described in the Examples below. Experiments performed using murine models are predictive of the human response because of the similarities in immune responses between mouse and human. The GVL effect for example, is well documented in both murine and human systems. The results of these assays are predictive of the biological responses in vivo, in the DLI treated host.

From the results of tests in animal models, the concentrations of compound determined to be effective will be tested on a representative number of patients and the most effective concentration of compound that results in alleviation of the patient's disease will be used. Conditions which work for the majority of patients will be most preferred. The patients will generally be post BM transplant patients.

From the results of such assays and tests, it will be possible to determine optimum treatment conditions that will generate a population of leukocytes that are incapable of proliferation and GVHD but effective to promote destruction of a diseased cell or pathogen. The treated leukocyte population should have GVL activity.

#### Selection of leukocyte donor

For DLI, the donor has to be allogeneic with respect to the host (recipient) of the leukocyte infusion. HLA-A and -B are HLA I genes and HLA-DR is a HLA II gene. Each of these genes exist in multiple different allelic forms. Since each individual inherits 2 copies of chromosome 6 (containing HLA genes), the individual can express up to 6 different HLA-A, -B and -DR proteins (products of 2 different alleles at each locus). Preferably, the allogeneic donor is identical genotypically in 3 or more of the 6 HLA loci of HLA-A, B, and DR. In "haploidentical" transplantation, the donor and host are matched in 3 of the 6 HLA loci. Ideally, the donor and recipient are identical for HLA-A, B and DRB1. Unrelated HLA-matched donors can be searched through the U.S. National Marrow Donor Program (NMPD).

Currently, for patients up to the age of 55 years who receive unmodified non-T cell depleted graft, the patient's and donor's HLA-A, B and DRB1 genes have to be identical. A patient who is 36 years of age or younger may be transplanted from a donor who differs by no more than one minor antigen mismatch for HLA-A, -B or -DR. An HLA-A or B



minor mismatch is defined as 2 antigens that belong to the same cross reactive group. An HLA-DR minor mismatch is defined as two haplotypes that express the same DR specificity but differ for DRB1 alleles. For criteria for suitable allogeneic donors, see O'Reilly, R. et al. Allogeneic Marrow Transplants: Approaches For The Patient Lacking A Donor, in Hematology-1996, The Education Program of the American Society of Hematology, pp. 132-146.

#### Preparation of leukocytes

The starting material for the leukocyte populations of the present invention can be provided, for example, by regional blood processing service centers similar to those currently in existence for the processing of peripheral blood stem cells, as well as other sources of hematopoietic stem cells, such as bone marrow. Leukocytes from the donor can be apheresed (apheresis can be done at a variety of facilities including the local blood center or hospital blood bank) and treated at any appropriate facility equipped to perform the treatment. Alternatively, apheresed leukocytes can be shipped overnight to a Good Manufacturing Practice (GMP) cell processing facility for treatment and quality assurance testing. The treated leukocytes for DLI can then be sent by same day delivery to the patient's hospital for administration to the patient. Alternatively, the treated leukocytes can be cryopreserved using methods known in the art such as by control rate freezing in 10% DMSO and storage in liquid nitrogen (see Russel et al. Bone Marrow Transpl. 19:861-866, 1997). When needed for transfusion, the frozen leukocytes will be thawed and washed to remove the DMSO.

One conventional method for the preparation of purified leukocytes involves isolation from whole blood by density gradient centrifugation. This typically involves the isolation of T cell-enriched white blood cells by centrifugation through a Ficoll gradient. See e.g. Longley and Stewart, J. Immunol. Methods, 121, 33-38, 1989. This procedure is carried out as follows: (1) freshly drawn blood samples (e.g. 50 - 200 milliliters) are layered over Ficoll carefully so that the interface is undisturbed; (2) the leukocytes are collected (after centrifugation) by removing the opaque band of cells located at the gradient interface and, (3) the collected cells are washed free of Ficoll. The red blood cells and granulocytes pellet in this system. To remove Ficoll, the cells are usually washed one or more times by centrifuging and removing the supernatant. This procedure yields a

purified leukocyte preparation, *i.e.* a preparation that is substantially free of red blood cells.

Automated methods exist for preparing large preparations of leukocytes. For example, the present invention contemplates processing blood as done by a leukapheresis machine (e.g., COBE Spectra Apheresis System, COBE BCT, Inc. Lakewood, CO) according to the manufacturer's directions. Preferably, an apheresis machine that provides a leukocyte preparation with the lowest percentage hematocrit is used. After leukapheresis, the leukocyte preparation can then be washed and diluted with the appropriate solution (plasma, infusion grade solution, etc. as described above) to achieve a final hematocrit of less than 0.5%. Percoll separation can be applied as a further purification step if the above procedure was insufficient to reduce the hematocrit levels. As an alternative to leukapheresis, red cell removal methods are well-known to those of skill in the art.

If leukocyte populations containing only substantially T cells and NK cells or other combination of leukocytes are desired, selected cell subsets can be isolated from the leukocyte preparation, e.g., by using fluorescence activated cell sorting (FACS) using positive and/or negative selection with the appropriate antibodies that identify specific cell surface markers. Examples of surface markers that can be used for selection include, but are not limited to, CD4, CD8, CD16 and CD56. Such cell separation procedures are well known in the art, see, e.g. Ed Harlow and David Lane, Antibodies A laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988.

Purified leukocyte populations are suspended in the appropriate solution as disclosed above. The purified leukocyte populations can be treated immediately or cryopreserved for later treatment. In preparation for proliferation inactivation, the purified, mixed leukocyte preparation is resuspended in an isotonic solution such as plasma, synthetic media, or a combination of the two. The purified leukocyte population will generally be prepared at a cell density of  $10$  to  $10^9$  cells per ml, preferably  $10^4$  to  $10^7$  cells/ml, ideally  $2 \times 10^6$  cells/ml.

The invention contemplates various scenarios for the preparation and administration or use of the treated leukocytes. The peripheral blood (or alternative leukocyte source including bone marrow or cord blood) can be collected and frozen until ready for proliferation inactivation treatment at which time the cells are thawed and washed free of the cryopreservative, optionally processed to obtain purified leukocyte

preparations, and subjected to treatment. The treated leukocyte populations can subsequently be used to infuse a patient. The following alternative scenarios are also possible: i) collection of peripheral blood, processing, leukocyte treatment, and infusion; ii) collection, processing, leukocyte treatment, freezing of treated cells, washing off of cryopreservative, and infusion; iii) vaccination of donor (one or more times) with a target antigen to expand antigen-specific T cells, collection of peripheral blood from vaccinated donor, processing, treatment, and infusion; iv) in vitro sensitization of donor leukocytes with a target antigen to expand antigen-specific T cells, leukocyte treatment, and infusion.

## Compounds

In one embodiment of the invention, compounds capable of forming a covalent bond with a nucleic acid are suitable for the purposes of achieving the leukocyte population of the present invention. These compounds preferably include a moiety which binds non-covalently with a nucleic acid, as well as the same or different moiety which is capable of reacting to form a covalent bond with a nucleic acid. The compound will preferably have the following properties: i) high binding affinity for nucleic acid; ii) solubility in aqueous solution; and iii) ability to penetrate the leukocyte membrane. Moieties capable of forming a covalent bond with nucleic acid include chemically reactive moieties, which do not require an outside stimulation in order to be activated and react with a nucleic acid, and photoactivatable moieties, which react with nucleic acids only after stimulus in the form of electromagnetic radiation.

As used herein, the term "alkylator compound" refers to a compound including at least one chemically reactive moiety capable of reacting to form a covalent bond with a nucleic acid, in the absence of an external stimulus, such as light stimulation.

The compound capable of forming a covalent bond with a nucleic acid further may be photoactivatable. The "photoactivatable compound", as defined herein, includes a moiety which is capable of forming a covalent bond with a nucleic acid upon photoactivation by stimulus of light of a certain wavelength.

Preferably, the compound includes both a moiety capable of reacting to form a covalent bond with a nucleic acid, and a moiety capable of binding non-covalently with a nucleic acid. Within the scope of the invention, the moiety which is capable of binding with a nucleic acid also may serve as the moiety which is capable of covalently reacting with the nucleic acid, and may be, for example, photoactivatable.

In one embodiment, the alkylator compound comprises: a nucleic acid binding moiety; a moiety capable of reacting to form a covalent bond with nucleic acid ("referred to herein as an effector moiety"); and a frangible linker covalently linking the nucleic acid moiety and the effector moiety. In a preferred embodiment, in aqueous solution, at appropriate pH values, these compounds have a period of activity during which they can bind to and react with nucleic acid. After this period, the compounds break down to products which are no longer able to bind well to nor react with nucleic acid.

The chemical organization of these alkylator compounds can be broadly described as an anchor, covalently bonded to a frangible linker, which is covalently bonded to an effector. "Anchor", also referred to as "nucleic acid binding moiety" is defined as a moiety which binds non-covalently to a nucleic acid biopolymer (DNA, RNA, or synthetic analogues thereof). "Effector" or "Effector moiety" is defined as a moiety which reacts with nucleic acid by a mechanism which forms a covalent bond with the nucleic acid. "Frangible linker" is defined as a moiety which serves to covalently link the anchor and effector, and which will degrade under certain conditions so that the anchor and effector are no longer linked covalently. The anchor-frangible linker-effector arrangement enables the compounds to bind specifically to nucleic acid (due to the anchor's binding ability). This brings the effector into proximity for reaction with the nucleic acid.

Preferably, the nucleic acid binding moiety is selected from the group consisting of aromatic intercalators, acridines, acridine derivatives, ellipticenes, 2- polyamines, groove binders, and hydrophobic or shape selective binders. In preferred embodiments, the frangible linker comprises a functional unit selected from the group consisting of forward esters, reverse esters, forward thioesters, reverse thioesters, forward and reverse thionoesters, forward and reverse dithioic acids, sulfates, forward and reverse sulfonates, phosphates, and forward and reverse phosphonate groups. The effector moiety preferably comprises a functional group selected from the group consisting of mustard groups, mustard group equivalents, epoxides, aldehydes, and formaldehyde synthons.

When the alkylator compound is combined with a leukocyte composition at physiological pH, to form a reaction mixture, the effector portion of the compound reacts with nucleic acid present with which it comes into contact. Effector moieties which do not react with nucleic acid are gradually hydrolyzed by the solvent. Hydrolysis of the frangible linker occurs concurrently with the effector-nucleic acid reaction and effector hydrolysis. It is desirable that the frangible linker break down at a rate slow enough to

5 permit inactivation of leukocyte proliferation; that is, the rate of breakdown of the frangible linker is slower than the rate at which the compound reacts with leukocyte nucleic acid. After a sufficient amount of time has passed, the compound has broken down into the anchor (which may also bear fragments of the frangible linker) and the effector-nucleic acid breakdown products (where fragments of the frangible linker may also remain attached to the effector), or into the anchor (which may also bear fragments of the frangible linker) and the hydrolyzed effector breakdown products (where fragments of the frangible linker may also remain attached to the effector). Additional fragments of the frangible linker may also be generated upon degradation of the compound which do not remain bonded to either the anchor or the effector. The exact embodiment of the alkylator compound of the invention determines whether the anchor breakdown product or the effector breakdown product bears fragments of the frangible linker, or whether additional fragments of the frangible linker are generated which do not remain bonded to either the anchor or the effector breakdown products.

15 Preferred alkylator compounds are compounds which, upon cleavage of the frangible linker, result in breakdown products of low mutagenicity. Mutagenicity of the compounds, after hydrolysis of the effector, is due primarily to the anchor moiety, as the anchor interacts with nucleic acid and may have the potential to interfere with nucleic acid replication, even if the effector moiety has been hydrolyzed. After cleavage of the frangible linker, the anchor fragment has substantially reduced mutagenicity.

20 In additional embodiments of the invention, the compound capable of forming a covalent bond with nucleic acid is removed from the reaction mixture after a certain period of time. The optimal reaction time can be determined empirically, as described *infra* in the Examples. Procedures and compositions for removal of compounds from the reaction mixture are provided in co-owned U.S. Patent Applications Serial Nos. 08/779,830; 08/779,885; and 09/003,113; and in co-owned U.S. Patent Applications Attorney Docket Nos. 28217-20004.21 and 28217-20004.22, both filed July 8, 1998. The disclosures of all of the patent applications mentioned in the preceding sentence are hereby incorporated herein by reference in their entireties. Alternatively, quenching agents which react with, and inactivate the compounds can be added to the reaction mixture. Exemplary quenching agents and methods are disclosed in co-owned U. S. Provisional Patent Application No. 60/070,597, filed January 6, 1998 and U.S. Patent Application attorney docket No. 28217-

20006.00, filed July 6, 1998; the disclosures of which are hereby incorporated herein by reference in their entirety.

A wide variety of groups are available for use as the anchors, linkers, and effectors. Examples of anchor groups which can be used in the alkylator compound include, but are not limited to, intercalators (including aromatic intercalators), minor groove binders, major groove binders, molecules which bind by electrostatic interactions or hydrophobic interactions, and molecules which bind by sequence specific interactions. The following is a non-limiting list of possible anchor groups:

acridines (and acridine derivatives, e.g. proflavine, acriflavine, diacridines, acridones, benzacridines, quinacrines), actinomycins, anthracyclines, rhodomycins, daunomycin, thioxanthenones (and thioxanthene derivatives, e.g. miracil D), anthramycin, mitomycins, echinomycin (quinomycin A), triostins, ellipticine (and dimers, trimers and analogs thereof), norphilin A, fluorenes (and derivatives, e.g. fluorenones, fluorenodiamines), phenazines, phenanthridines, phenothiazines (e.g., chlorpromazine), phenoxazines, benzothiazoles, xanthenes and thioxanthenes, anthraquinones, anthrapyrazoles, benzothiopyranoidoles, 3,4-benzopyrene, 1-pyrenyloxirane, benzanthraces, benzodipyrones, quinolines (e.g., chloroquine, quinine, phenylquinoline carboxamides), furocoumarins (e.g., psoralens and isopsoralens), ethidium, propidium, coralyne, and polycyclic aromatic hydrocarbons and their oxirane derivatives; distamycin, netropsin, other lexitropsins, Hoechst 33258 and other Hoechst dyes, DAPI (4',6-diamidino-2-phenylindole), berenil, and triarylmethane dyes; aflatoxins; spermine, spermidine, and other polyamines; and

nucleic acids or analogs which bind by sequence specific interactions such as triple helix formation, D-loop formation, and direct base pairing to single stranded targets. Derivatives of these compounds are also non-limiting examples of anchor groups, where a derivative of a compound includes, but is not limited to, a compound which bears one or more substituent of any type at any location, oxidation or reduction products of the compound, etc.

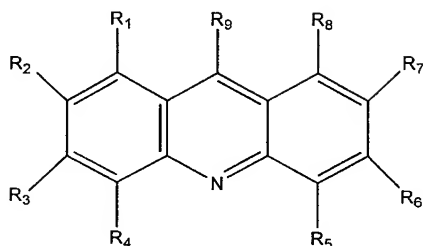
Examples of linkers which can be used in the invention are, but are not limited to, compounds which include functional groups such as ester (where the carbonyl carbon of the ester is between the anchor and the  $sp^3$  oxygen of the ester; this arrangement is also called "forward ester"), "reverse ester" (where the  $sp^3$  oxygen of the ester is between the

anchor and the carbonyl carbon of the ester), thioester (where the carbonyl carbon of the thioester is between the anchor and the sulfur of the thioester, also called "forward thioester"), reverse thioester (where the sulfur of the thioester is between the anchor and the carbonyl carbon of the thioester, also called "reverse thioester"), forward and reverse thionoester, forward and reverse dithioic acid, sulfate, forward and reverse sulfonates, phosphate, and forward and reverse phosphonate groups. "Thioester" designates the -C(=O)-S- group; "thionoester" designates the -C(=S)-O- group, and "dithioic acid" designates the -C(=S)-S- group. For groups which can be designated as "forward" and "reverse", the forward orientation is that orientation of the functional groups wherein, after hydrolysis of the functional group, the resulting acidic function is covalently linked to the anchor moiety and the resulting alcohol or thiol function is covalently linked to the effector moiety. The reverse orientation is that orientation of the functional groups wherein, after hydrolysis of the functional group, the resulting acidic function is covalently linked to the effector moiety and the resulting alcohol or thiol function is covalently linked to the anchor moiety.

Examples of effectors which can be used in the invention are, but are not limited to, mustard groups, mustard group equivalents, epoxides, aldehydes, formaldehyde synthons, and other alkylating and cross-linking agents. Mustard groups are defined as including mono or bis haloethylamine groups, and mono haloethylsulfide groups. Mustard group equivalents are defined by groups that react by a mechanism similar to the mustards (that is, by forming an aziridinium intermediate, or by having or by forming an aziridine ring, which can react with a nucleophile), such as mono or bis mesylethylamine groups, mono mesylethylsulfide groups, mono or bis tosylethylamine groups, and mono tosylethylsulfide groups. Formaldehyde synthons are defined as any compound that breaks down to formaldehyde in aqueous solution, including hydroxymethylamines such as hydroxymethylglycine. Examples of formaldehyde synthons are given in U.S. Pat. No. 4,337,269 and in International Patent Application WO 97/02028.

Alkylator compounds that can be used to prepare the leukocytes of this invention are described by the following general formulas I, II, and III.

General formula I is:



(I)

wherein at least one of R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub>, R<sub>6</sub>, R<sub>7</sub>, R<sub>8</sub> and R<sub>9</sub> is -V-W-X-E as defined below, and the remainder of R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, R<sub>4</sub>, R<sub>5</sub>, R<sub>6</sub>, R<sub>7</sub>, R<sub>8</sub> and R<sub>9</sub> are independently selected from the group consisting of -H, -R<sub>10</sub>, -O-R<sub>10</sub>, -NO<sub>2</sub>, -NH<sub>2</sub>, -NH-R<sub>10</sub>, -N(R<sub>10</sub>)<sub>2</sub>, -F, -Cl, -Br, -I, -C(=O)-R<sub>10</sub>, -C(=O)-O-R<sub>10</sub>, and -O-C(=O)-R<sub>10</sub>,

where -R<sub>10</sub> is independently H, -C<sub>1-8</sub> alkyl, -C<sub>1-8</sub> heteroalkyl, -aryl, -heteroaryl, -C<sub>1-3</sub>alkyl-aryl, -C<sub>1-3</sub>heteroalkyl-aryl, -C<sub>1-3</sub>alkyl-heteroaryl, -C<sub>1-3</sub>heteroalkyl-heteroaryl, -aryl-C<sub>1-3</sub>alkyl, -aryl-C<sub>1-3</sub>heteroalkyl, -heteroaryl-C<sub>1-3</sub>alkyl, -heteroaryl-C<sub>1-3</sub>heteroalkyl, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl, or -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl;

V is independently -R<sub>11</sub>-, -NH-R<sub>11</sub>- or -N(CH<sub>3</sub>)-R<sub>11</sub>-, where -R<sub>11</sub>- is independently -C<sub>1-8</sub>alkyl-, -C<sub>1-8</sub>heteroalkyl-, -aryl-, -heteroaryl-, -C<sub>1-3</sub>alkyl-aryl-, -C<sub>1-3</sub>heteroalkyl-aryl-, -C<sub>1-3</sub>alkyl-heteroaryl-, -C<sub>1-3</sub>heteroalkyl-heteroaryl-, -aryl-C<sub>1-3</sub>alkyl-, -aryl-C<sub>1-3</sub>heteroalkyl-, -heteroaryl-C<sub>1-3</sub>alkyl-, -heteroaryl-C<sub>1-3</sub>heteroalkyl-, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl-, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl-, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl-, or -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl-;

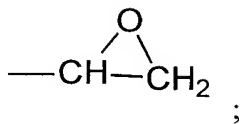
W is independently -C(=O)-O-, -O-C(=O)-, -C(=S)-O-, -O-C(=S)-, -C(=S)-S-, -S-C(=S)-, -C(=O)-S-, -S-C(=O)-, -O-S(=O)<sub>2</sub>-O-, -S(=O)<sub>2</sub>-O-, -O-S(=O)<sub>2</sub>-, -O-P(=O)(-OR<sub>10</sub>)-O-, -P(=O)(-OR<sub>10</sub>)-O-, -O-P(=O)(-OR<sub>10</sub>)-;

X is independently -R<sub>11</sub>-; and

E is independently selected from the group consisting of -N(R<sub>12</sub>)<sub>2</sub>, -N(R<sub>12</sub>)(R<sub>13</sub>), -S-R<sub>12</sub>,



and



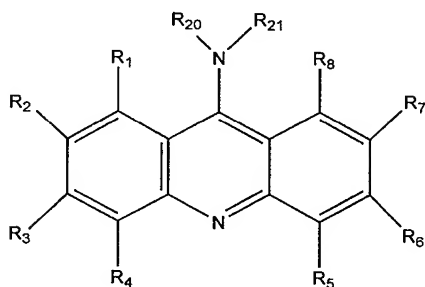
where  $\text{-R}_{12}$  is  $\text{-CH}_2\text{CH}_2\text{-G}$ , where each G is independently  $\text{-Cl}$ ,  $\text{-Br}$ ,  $\text{-I}$ ,  $\text{-O-S(=O)}_2\text{-CH}_3$ ,  $\text{-O-S(=O)}_2\text{-CH}_2\text{-C}_6\text{H}_5$ , or  $\text{-O-S(=O)}_2\text{-C}_6\text{H}_4\text{-CH}_3$ ;

and where  $\text{R}_{13}$  is independently  $\text{-C}_{1-8}$  alkyl,  $\text{-C}_{1-8}$  heteroalkyl, -aryl, -heteroaryl,  $\text{-C}_{1-3}$ alkyl-aryl,  $\text{-C}_{1-3}$ heteroalkyl-aryl,  $\text{-C}_{1-3}$ alkyl-heteroaryl,  $\text{-C}_{1-3}$ heteroalkyl-heteroaryl, -aryl- $\text{-C}_{1-3}$ alkyl, -aryl- $\text{-C}_{1-3}$ heteroalkyl, -heteroaryl- $\text{-C}_{1-3}$ alkyl, -heteroaryl- $\text{-C}_{1-3}$ heteroalkyl,  $\text{-C}_{1-3}$ alkyl-aryl- $\text{-C}_{1-3}$  alkyl,  $\text{-C}_{1-3}$ heteroalkyl-aryl- $\text{-C}_{1-3}$  alkyl,  $\text{-C}_{1-3}$ alkyl-heteroaryl- $\text{-C}_{1-3}$  alkyl,  $\text{-C}_{1-3}$ alkyl-aryl- $\text{-C}_{1-3}$  heteroalkyl,  $\text{-C}_{1-3}$ heteroalkyl-heteroaryl- $\text{-C}_{1-3}$  alkyl,  $\text{-C}_{1-3}$ heteroalkyl-aryl- $\text{-C}_{1-3}$  heteroalkyl,  $\text{-C}_{1-3}$ alkyl-heteroaryl- $\text{-C}_{1-3}$  heteroalkyl, or  $\text{-C}_{1-3}$ heteroalkyl-heteroaryl- $\text{-C}_{1-3}$  heteroalkyl;

and all salts and stereoisomers (including enantiomers and diastereomers) thereof.

A preferred composition according to Formula I is the compound S-303, wherein V is  $\text{-NHR}_{11}\text{-}$ ,  $\text{R}_{11}$  is  $\text{-CH}_2\text{CH}_2\text{-}$ , W is  $\text{-C(=O)-O-}$ , X is  $\text{-CH}_2\text{CH}_2\text{-}$ , E is  $\text{-N(R}_{12})_2\text{-}$ ,  $\text{R}_{12}$  is  $\text{-CH}_2\text{CH}_2\text{G}$ , and G is  $\text{-Cl}$ . See also co-owned PCT application US98/00531.

General formula II is:



(II)

where  $\text{R}_1$ ,  $\text{R}_2$ ,  $\text{R}_3$ ,  $\text{R}_4$ ,  $\text{R}_5$ ,  $\text{R}_6$ ,  $\text{R}_7$ , and  $\text{R}_8$  are independently selected from the group consisting of  $\text{-H}$ ,  $\text{-R}_{10}$ ,  $\text{-O-R}_{10}$ ,  $\text{-NO}_2$ ,  $\text{-NH}_2$ ,  $\text{-NH-R}_{10}$ ,  $\text{-N(R}_{10})_2$ ,  $\text{-F}$ ,  $\text{-Cl}$ ,  $\text{-Br}$ ,  $\text{-I}$ ,  $\text{-C(=O)-R}_{10}$ ,  $\text{-C(=O)-O-R}_{10}$ , and  $\text{-O-C(=O)-R}_{10}$ ,

where  $\text{-R}_{10}$  is independently H,  $\text{-C}_{1-8}$  alkyl,  $\text{-C}_{1-8}$  heteroalkyl, -aryl, -heteroaryl,  $\text{-C}_{1-3}$ alkyl-aryl,  $\text{-C}_{1-3}$ heteroalkyl-aryl,  $\text{-C}_{1-3}$ alkyl-heteroaryl,  $\text{-C}_{1-3}$ heteroalkyl-heteroaryl, -aryl- $\text{-C}_{1-3}$ alkyl, -aryl- $\text{-C}_{1-3}$ heteroalkyl, -heteroaryl- $\text{-C}_{1-3}$ alkyl, -heteroaryl- $\text{-C}_{1-3}$ heteroalkyl,  $\text{-C}_{1-3}$ alkyl-aryl- $\text{-C}_{1-3}$  alkyl,  $\text{-C}_{1-3}$ heteroalkyl-aryl- $\text{-C}_{1-3}$  alkyl,  $\text{-C}_{1-3}$ alkyl-heteroaryl- $\text{-C}_{1-3}$  alkyl,

-C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl,  
 -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl, or  
 -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl;

R<sub>20</sub> is -H or -CH<sub>3</sub>; and

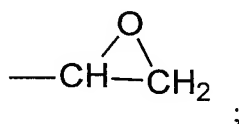
R<sub>21</sub> is -R<sub>11</sub>-W-X-E,

where -R<sub>11</sub>- is independently -C<sub>1-8</sub>alkyl-, -C<sub>1-8</sub>heteroalkyl-, -aryl-, -heteroaryl-,  
 -C<sub>1-3</sub>alkyl-aryl-, -C<sub>1-3</sub>heteroalkyl-aryl-, -C<sub>1-3</sub>alkyl-heteroaryl-, -C<sub>1-3</sub>heteroalkyl-heteroaryl-,  
 -aryl-C<sub>1-3</sub>alkyl-, -aryl-C<sub>1-3</sub>heteroalkyl-, -heteroaryl-C<sub>1-3</sub>alkyl-, -heteroaryl-C<sub>1-3</sub>heteroalkyl-,  
 -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub>  
 alkyl-, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl-, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl-,  
 -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl-, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl-, or  
 -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl-;

W is independently -C(=O)-O-, -O-C(=O)-, -C(=S)-O-, -O-C(=S)-, -C(=S)-S-,  
 -S-C(=S)-, -C(=O)-S-, -S-C(=O)-, -O-S(=O)<sub>2</sub>-O-, -S(=O)<sub>2</sub>-O-, -O-S(=O)<sub>2</sub>-,  
 -O-P(=O)(-OR<sub>10</sub>)-O-, -P(=O)(-OR<sub>10</sub>)-O-, -O-P(=O)(-OR<sub>10</sub>)-;

X is independently -R<sub>11</sub>-; and

E is independently selected from the group consisting of -N(R<sub>12</sub>)<sub>2</sub>, -N(R<sub>12</sub>)(R<sub>13</sub>),  
 -S-R<sub>12</sub>,  
 and

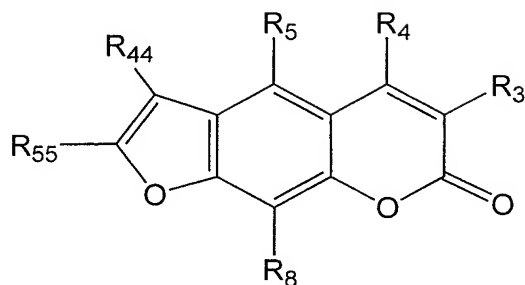


where -R<sub>12</sub> is -CH<sub>2</sub>CH<sub>2</sub>-G, where each G is independently -Cl, -Br, -I,  
 -O-S(=O)<sub>2</sub>-CH<sub>3</sub>, -O-S(=O)<sub>2</sub>-CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>, or -O-S(=O)<sub>2</sub>-C<sub>6</sub>H<sub>4</sub>-CH<sub>3</sub>;

and where R<sub>13</sub> is independently -C<sub>1-8</sub> alkyl, -C<sub>1-8</sub> heteroalkyl, -aryl, -heteroaryl,  
 -C<sub>1-3</sub>alkyl-aryl, -C<sub>1-3</sub>heteroalkyl-aryl, -C<sub>1-3</sub>alkyl-heteroaryl, -C<sub>1-3</sub>heteroalkyl-heteroaryl,  
 -aryl-C<sub>1-3</sub>alkyl, -aryl-C<sub>1-3</sub>heteroalkyl, -heteroaryl-C<sub>1-3</sub>alkyl, -heteroaryl-C<sub>1-3</sub>heteroalkyl,  
 -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> alkyl,  
 -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl,  
 -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl, or  
 -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl;

and all salts and stereoisomers (including enantiomers and diastereomers) thereof.

General formula III is:



(III)

wherein at least one of  $R_{44}$ ,  $R_{55}$ ,  $R_3$ ,  $R_4$ ,  $R_5$ , and  $R_8$  is -V-W-X-E, and the remainder of  $R_{44}$ ,  $R_{55}$ ,  $R_3$ ,  $R_4$ ,  $R_5$ , and  $R_8$  are independently selected from the group consisting of -H, - $R_{10}$ , -O- $R_{10}$ , -NO<sub>2</sub>, -NH<sub>2</sub>, -NH- $R_{10}$ , -N( $R_{10}$ )<sub>2</sub>, -F, -Cl, -Br, -I, -C(=O)- $R_{10}$ , -C(=O)-O- $R_{10}$ , and -O-C(=O)- $R_{10}$ ,

where - $R_{10}$  is independently H, -C<sub>1-8</sub> alkyl, -C<sub>1-8</sub> heteroalkyl, -aryl, -heteroaryl, -C<sub>1-3</sub>alkyl-aryl, -C<sub>1-3</sub>heteroalkyl-aryl, -C<sub>1-3</sub>alkyl-heteroaryl, -C<sub>1-3</sub>heteroalkyl-heteroaryl, -aryl-C<sub>1-3</sub>alkyl, -aryl-C<sub>1-3</sub>heteroalkyl, -heteroaryl-C<sub>1-3</sub>alkyl, -heteroaryl-C<sub>1-3</sub>heteroalkyl, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl, or -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl;

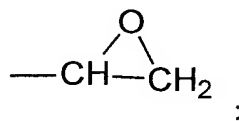
V is independently - $R_{11}$ -, -NH- $R_{11}$ - or -N(CH<sub>3</sub>)- $R_{11}$ -, where - $R_{11}$ - is independently -C<sub>1-8</sub>alkyl-, -C<sub>1-8</sub>heteroalkyl-, -aryl-, -heteroaryl-, -C<sub>1-3</sub>alkyl-aryl-, -C<sub>1-3</sub>heteroalkyl-aryl-, -C<sub>1-3</sub>alkyl-heteroaryl-, -C<sub>1-3</sub>heteroalkyl-heteroaryl-, -aryl-C<sub>1-3</sub>alkyl-, -aryl-C<sub>1-3</sub>heteroalkyl-, -heteroaryl-C<sub>1-3</sub>alkyl-, -heteroaryl-C<sub>1-3</sub>heteroalkyl-, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl-, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl-, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl-, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl-, or -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl-;

W is independently -C(=O)-O-, -O-C(=O)-, -C(=S)-O-, -O-C(=S)-, -C(=S)-S-, -S-C(=S)-, -C(=O)-S-, -S-C(=O)-, -O-S(=O)<sub>2</sub>-O-, -S(=O)<sub>2</sub>-O-, -O-S(=O)<sub>2</sub>-, -O-P(=O)(-OR<sub>10</sub>)-O-, -P(=O)(-OR<sub>10</sub>)-O-, -O-P(=O)(-OR<sub>10</sub>)-;

X is independently - $R_{11}$ -; and

E is independently selected from the group consisting of -N(R<sub>12</sub>)<sub>2</sub>, -N(R<sub>12</sub>)(R<sub>13</sub>),  
-S-R<sub>12</sub>,

and



where -R<sub>12</sub> is -CH<sub>2</sub>CH<sub>2</sub>-G, where each G is independently -Cl, -Br, -I,  
-O-S(=O)<sub>2</sub>-CH<sub>3</sub>, -O-S(=O)<sub>2</sub>-CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>, or -O-S(=O)<sub>2</sub>-C<sub>6</sub>H<sub>4</sub>-CH<sub>3</sub>;

and where R<sub>13</sub> is independently -C<sub>1-8</sub> alkyl, -C<sub>1-8</sub> heteroalkyl, -aryl, -heteroaryl,  
-C<sub>1-3</sub>alkyl-aryl, -C<sub>1-3</sub>heteroalkyl-aryl, -C<sub>1-3</sub>alkyl-heteroaryl, -C<sub>1-3</sub>heteroalkyl-heteroaryl,  
-aryl-C<sub>1-3</sub>alkyl, -aryl-C<sub>1-3</sub>heteroalkyl, -heteroaryl-C<sub>1-3</sub>alkyl, -heteroaryl-C<sub>1-3</sub>heteroalkyl,  
-C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> alkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> alkyl,  
-C<sub>1-3</sub>alkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> alkyl,  
-C<sub>1-3</sub>heteroalkyl-aryl-C<sub>1-3</sub> heteroalkyl, -C<sub>1-3</sub>alkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl, or  
-C<sub>1-3</sub>heteroalkyl-heteroaryl-C<sub>1-3</sub> heteroalkyl;

and all salts and stereoisomers (including enantiomers and diastereomers) thereof.

It will be appreciated that, in general formula I above, the acridine nucleus is the  
anchor moiety, the -V-W-X- group(s) comprises the frangible linker, and the E group(s) is  
the effector group. Similarly, in general formula III above, the psoralen nucleus is the  
anchor moiety, the -V-W-X- group(s) comprises the frangible linker, and the E group(s) is  
the effector group. General formula II is a subset of general formula I.

Examples 7-12 illustrate the synthesis of these compounds to produce the  
leukocytes of the invention.

In one embodiment of the invention, 4'-(4-amino-2-oxa) butyl-4,5',8-  
trimethylpsoralen ("S-59"), previously disclosed in co-owned U.S. Patent No. 5,399,719  
(the disclosure of which is hereby incorporated by reference herein in its entirety), is also  
useful for photochemical treatment of leukocyte populations.

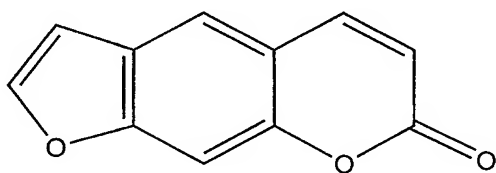
Photoactivatable compounds suitable for use in the methods of this invention  
include: Furocoumarins; Actinomycins; Anthracyclinones; Anthramycins;  
Benzodipyrones; Fluorenes and Fluorenones; Monostral Fats Blue; Norphillin A; Organic  
Dyes; Phenanthridines; Phenazathionium Salts; Phenazines; Phenothiazines; Phenylazides;

Quinolines and Thioxanthenones. The preferred species of photoactivatable compounds described herein is commonly referred to as the furocoumarins.

#### Psoralen and derivatives

5 Psoralens are planar, aromatic organic compounds belonging to the group of furocoumarins. Psoralens are found in nature, principally in plants, including limes, cloves, celery, parsnips and figs. In humans, psoralens have been used in photochemotherapy for the management of vitiligo, psoriasis and mycosis fungoides. For a review of psoralen photochemistry, see Parsons, B.J. Photochem. Photobiol. 32:813-821, 10 1980. The basic structure of psoralen is shown below.

In particular, the present invention contemplates psoralens, [7H-furo(3,2-g)-(1)-benzopyran-7-one, or b-lactone of 6-hydroxy-5-benzofuranacrylic acid], which are linear:



15 and in which the two oxygen residues appended to the central aromatic moiety have a 1, 3 orientation, and further in which the furan ring moiety is linked to the 6 position of the two ring coumarin system. Psoralen derivatives are derived from substitution of the linear furocoumarin at the 3, 4, 5, 8, 4', or 5' positions.

20 With regard to safety concerns, psoralen is already present in our diets. Psoralens are relatively inactive in the absence of long-wavelength UVA radiation. The effective half-life of UVA-activated psoralen is measured in milliseconds. Any drug remaining after radiation returns to the inactive state, thus limiting side effects.

25 Previous protocols for inactivation of pathogens have necessitated the removal of molecular oxygen from the reaction before and during exposure to light, to prevent damage to blood products from oxygen radicals produced during irradiation. See L. Lin et al., Blood 74:517 (1989); US Patent No. 4,727,027, to Wieschahn. The methods of the present invention can be used to arrest proliferation in leukocytes in the presence of

oxygen. Further, with novel psoralens used in the methods of the present invention, there is no need to reduce the concentration of molecular oxygen. The preferred psoralens have low mutagenicity and high nucleic acid binding affinity.

8-Methoxypsoralen (known in the literature under various names, e.g., xanthotoxin, methoxsalen, 8-MOP) is a naturally occurring psoralen with low mutagenicity in the Ames assay.

4'-Aminomethyl-4,5',8-trimethylpsoralen (AMT) is one of most reactive nucleic acid binding psoralen derivatives, providing up to 1 AMT adduct per 3.5 DNA base pairs (S.T. Isaacs, G. Wieseahn and L.M. Hallick, NCI Monograph 66:21, 1984).

"4'-primaryamino-substituted psoralens", or "4-PAP", are defined as psoralen compounds which have an  $\text{NH}_2$  group linked to the 4'-position of the psoralen by a hydrocarbon chain having a total length of 2 to 20 carbons, where 0 to 6 of those carbons are independently replaced by NH or O, and each point of replacement is separated from each other point of replacement by at least two carbons, and is separated from the psoralen by at least one carbon. 4'-primaryamino-substituted psoralens may have additional substitutions on the 4, 5', and 8 positions of the psoralen, said substitutions include, but are not limited to, the following groups: H and  $(\text{CH}_2)_n\text{CH}_3$ , where  $n = 0-6$ .

"5'-primaryamino-substituted psoralens", also referred to herein by the acronym "5-PAP", are defined as psoralen compounds which have an  $\text{NH}_2$  group linked to the 5'-position of the psoralen by a hydrocarbon chain having a total length of 1 to 20 carbons, where 0 to 6 of those carbons are independently replaced by NH or O, and each point of replacement is separated from each other point of replacement by at least two carbons, and is separated from the psoralen by at least one carbon. 5'-primaryamino-substituted psoralens may have additional substitutions on the 4, 4', and 8 positions of the psoralen, said substitutions include, but are not limited to, the following groups: H and  $(\text{CH}_2)_n\text{CH}_3$ , where  $n = 0-6$ . Examples of 4-PAP and 5-PAP (including S-59) as well as their methods of synthesis are disclosed in U.S. Patents Nos. 5,585,503; 5,578,736; 5,556,993; and 5,399,719.

Preferred psoralens include 5'-primaryamino-substituted psoralens and 4'-primaryamino-substituted psoralens (collectively referred to herein as PAP), 8-methoxy psoralen (8-MOP), 4'-aminomethyl 4,5',8-trimethylpsoralen (AMT), 5-methoxy psoralen (5-MOP), and trioxalen 4, 5' 8-trimethylpsoralen. AMT, 5-MOP, 8-MOP, and trioxalen are commercially available.

The most preferred psoralen, is S-59 which has the formula shown above (see under Abbreviations, S-59). S-59 is a synthetic psoralen that can bind reversibly to nucleic acids by intercalation. Upon illumination with UVA, intercalated S-59 forms monoadducts and interstrand crosslinks with RNA and DNA. S-59 photochemical treatment is nucleic acid specific and S-59 readily penetrates cellular and nuclear membranes. S-59 combines a higher water solubility and nucleic acid binding affinity, with a high quantum yield for the formation of nucleic acid adducts. This allows the use of lower psoralen concentrations for PCT for inhibiting proliferation in the absence of non-specific cellular damage. Mutagenicity and gene toxicity studies on S-59 demonstrated a 40,000–67,000 fold safety margin for the concentration used to decontaminate pathogens in blood products. Finally, the doses of S-59 used for PCT are well below the average daily dietary intake (Wagstaff, D.J. Reg. Tox. Pharm. 14:261–272, 1991) of furocoumarins, the chemical group that includes psoralens.

In one embodiment, a cell population comprising a population of leukocytes is treated with a photoactivatable compound including a moiety capable of forming a covalent bond with a nucleic acid upon photoactivation. Thus, to form a covalent bond with nucleic acid, the photoactivatable compound must be activated by light. Light encompasses ultraviolet light (UVA, UVB, UVC) and visible light. In one embodiment wavelengths used for PCT range from 200-450 nm. In a preferred embodiment, the light used for PCT is in the wavelength of 320-400 nm.

Preferably, the cell population is photochemically treated with UV light having a wavelength in the range of 320 to 400 nm. UVA radiation alone causes minimal damage to cells.

In one embodiment, the photoactivatable compound comprises a psoralen molecule. PCT with the appropriate concentration of psoralen and dose of UVA light will result in proliferation inhibition with the retention of leukocyte immunogenic functions effective, for example, to promote destruction of a diseased cell, an infected cell, or a pathogen, induce an antileukemic effect, facilitate engraftment by a second population of cells, promote immune reconstitution, facilitate immunotherapy, and treat mixed chimerism. Psoralen reactivity is not only specifically directed towards the nucleic acids versus the proteins in a cell, but there is also a differential binding affinity of psoralens for DNA vs RNA molecules due to the difference in nucleic acid structure that allows better intercalation in DNA (Cimino, G.D. *et al.*, Ann. Rev. Biochem. 54:1151–1193, 1985).

Additionally, using this technology, it may be possible to differentially modify transcriptionally active (vs. inactive) genes, due to their different conformation and state of histone binding which will affect psoralen binding. Treatments such as PCT therefore have the ability to interfere with cellular function, primarily at the level of DNA synthesis (replication), with a lesser effect on transcription, and minimal or no effect on protein synthesis. In the case of PCT, for example, the level of interference will be determined by the concentration of the psoralen and dose of UVA light used.

Samples of leukocytes mixed with a photoactivatable compound are photochemically treated using a photoactivation device. In general, a photoactivation device suitable for the present methods of PCT comprises the following: (a) a means for providing appropriate wavelengths of electromagnetic radiation to cause photoactivation of a photoactivatable compound; (b) means for supporting a plurality of samples in a fixed relationship with the radiation providing means during photoactivation; and (c) means for maintaining the temperature of the samples within a desired temperature range during photoactivation.

In one embodiment, the photoactivation device is capable of emitting a given intensity of a spectrum of electromagnetic radiation comprising wavelengths between 200 and 450 nm, preferably between 320 nm and 400 nm. Particular wavelengths can be selected using appropriate filters within the photoactivation device. A suitable photoactivation device as well as methods of photoactivating photoactivatable compounds by the use of the device are disclosed in U.S. Patent Nos. 5,184,020 and 5,503,721, both to Hearst et al. and in WO 96/39820. Other photoactivation devices that may be used include General Electric type F20T12-BLB fluorescent UVA bulbs (Alter, H. J., et al., The Lancet, 24:1446 (1988), and Type A405-TLGW/05 long wavelength ultraviolet lamp manufactured by P. W. Allen Co., London.

#### Photochemical treatment of leukocytes with psoralens

Stock solutions of psoralens are prepared in water or in the appropriate solvent as exemplified below in the Examples.

The stock psoralen solutions are added to suspensions of purified donor leukocyte populations to the desired final concentration. Units that were treated with 8-MOP were prepared by using PAS saturated with 8-MOP in the preparation procedure discussed above. The purified donor leukocytes are illuminated with UVA light to a final dose of



between  $10^{-3}$  to  $100 \text{ J/cm}^2$  in a photoactivation device. The samples are agitated during illumination. Samples of the leukocytes are taken prior to illumination as controls.

Typically, the PAP is added to the purified leukocyte preparation at a concentration of between  $10^{-4}$  and  $150 \text{ }\mu\text{M}$ , preferably between  $10^{-3}$  and  $150 \text{ }\mu\text{M}$ . The leukocytes will generally be at a cell density of between about  $10$  to  $10^9$  cells/ml, preferably between about  $10^3$  to  $10^8$  cells/ml, even more preferably between about  $10^4$  to  $10^7$  cells/ml, most preferably at about  $2 \times 10^6$  cells/ml.

In a preferred embodiment, PCT is performed on leukocytes combined with S-59 using ultraviolet light of wavelength of between  $320\text{-}400 \text{ nm}$ . Restricting the photoactivation process to wavelengths greater than  $320 \text{ nm}$  minimizes direct nucleic acid damage since there is very little absorption by nucleic acids above  $313 \text{ nm}$ . The cells will preferably receive a light dose in the range of about  $10^{-3}$  to  $100 \text{ J/cm}^2$ , more preferably  $1$  to  $3 \text{ J/cm}^2$ . In a most preferred embodiment, the light dose is at  $3 \text{ J/cm}^2$ . Illumination will generally be at a light intensity of between  $1$  to  $50 \text{ mW/cm}^2$ . The time period for exposure of the leukocyte sample to UV light will be between about  $1$  second to  $60$  minutes, preferably about  $3$  minutes, more preferably about one minute.

After PCT, the leukocyte samples will be assayed for effectiveness to proliferate and to participate in leukocyte activity, as described above and in the Examples. Primarily, the following parameters will be measured: cell viability; proliferation activity; cytokine secretion; and surface antigen expression.

#### Therapeutic applications

The treated leukocyte populations of the present invention, which are non-proliferating but retain immunological activity, have various prophylactic and therapeutic uses as described below. Typically, individuals receiving DLIs containing treated leukocyte populations will include but are not limited to, transplant patients such as post BM transplant patients or patients anticipating a solid organ transplant. The fact that the treated leukocytes do not elicit GVHD enables greater numbers of leukocytes (i.e. larger cell doses) to be administered in the DLI, thus maximizing the efficacy of treatment.

The treated leukocytes are useful for donor leukocyte infusion (DLI) especially to provide immunocompetence to allo-BMT patients, or immunocompromised patients such as elderly patients suffering from CLL. DLI using treated leukocytes are useful to alleviate various conditions including the following:

Leukemia including Chronic Myelogenous Leukemia (CML), Chronic Lymphocytic leukemia (CLL), Chronic myelomonocytic Leukemia (CmML), Acute Myelogenous Leukemia (AML), and Acute Lymphoblastic Leukemia (ALL); Multiple Myeloma (MM); Non-Hodgkin's lymphoma and Hodgkin's lymphoma; Large Cell Lymphoma (LCL); and  
5 Epstein-Barr virus induced B lymphoproliferative disorder (EBV-BLPD or EBV-induced lymphoma);

Solid tumors, including breast, lung, ovarian, testicular, prostate and colon cancer, melanoma, renal cell carcinoma, neuroblastoma, head and neck tumors;

Aplastic anemia; Myelodysplasia;

10 Immunodeficiencies e.g. Common variable, SCID, Wiskott-Aldrich syndrome, Agranulocytosis;

Mixed chimerism; and

Genetic diseases.

DLIs are useful in various formats for prophylactic or therapeutic purposes:

- 15 (a) adoptive immunotherapy for all allogeneic transplants;  
(b) immune reconstitution for immunosuppressed or immunodeficient patients;  
(c) treatment of elderly leukemia patients who are ineligible for transplant;  
(d) BMT light or minitransplants

The BMT light protocol is a two step process which involves: i) induction of  
20 transplantation tolerance using a minimum conditioning regimen and donor stem cells; and  
ii) immunotherapy with DLI repeatedly. This protocol circumvents the toxicity of chemotherapy regimens. The BMT light protocol is suitable in the following indications:  
for patients who are not normally eligible for transplants, particularly elderly patients (> 65 years old); and for CLL patients, most of whom are severely immunocompromised. BMT  
25 light regimen reduces toxicity, GVHD, and infection. An exemplary BMT light protocol is described in Example 13 below.

- (e) CD34 selected/T cell depleted allogeneic grafts plus concurrent DLI.

DLI provided concurrently with CD34+ selected cells (selection to enrich for stem and progenitor cells) are useful to confer adoptive immunotherapy against infections, such  
30 as by CMV, Epstein Barr virus (EBV), Adenovirus (Ad), Kaposi's Sarcoma associated Herpes virus, and fungal infections, and to treat leukemia, e.g., CML.

- (f) Treatment of mixed chimerism.  
(g) salvage chemotherapy or chemo-resistant leukemia

In one embodiment of the invention, PA-DLI is administered in haploidentical (haplo) transplantation. Normally, haplo transplantation requires almost complete T cell depletion, leading to severe, sustained immunosuppression. PA-DLI would be useful to maintain immunocompetence in the host; additionally, a GVL effect may be provided for the treatment of cancer.

In preferred embodiments, the leukocytes of the present invention that are incompetent in GVHD but retain some immune function will be used in DLI for the alleviation of relapsing cancer or removal of minimal residual disease following allogeneic BMT, particularly in CML and acute leukemias, lymphomas and multiple myelomas. The high number of donor CTLs specific to the bcr-abl protein of CML contributes to the efficacy of DLI in relapsing CML. A cancer patient is determined to be suffering a "relapse" if malignant cells are detected, by means routinely used by physicians to monitor the presence of cancerous cells.

In another embodiment, DLI containing the non-proliferating leukocytes is used in the treatment of leukemia patients during induction chemotherapy (initial round of chemotherapy) to avoid myeloablative regimens and to remove minimal residual disease. By using DLI in this setting, the toxic dose of chemotherapeutic drug given during induction could be reduced.

In one embodiment of the invention, PA-DLI can also be applied in solid organ transplants (e.g., kidney, heart, skin) as a means to increase the lifetime of allografts and the chances of donor organ acceptance. Most of the organ rejections in the allogeneic transplant occur due to the presence of passenger leukocytes in the donor organ. Due to the lack of proliferation and inability to mediate GVHD, the DLI (using leukocytes from the organ donor) may be used as a tolerization regimen before transplantation for the suppression of the immune response which will otherwise induce organ rejection. Tolerization of the host for solid organ transplant would consist of the following: before or during organ transplant procedure, infusion of PA-DLI with or without donor stem cells with some level of myeloablative regimen to allow donor cells to survive in circulation. DLI may facilitate engraftment of donor stem cells in this setting, allowing for mixed chimerism in the host.

In all types of transplant and engraftment situations, treated leukocytes can be administered to the host before, at the same time as, or after the transplant or graft. Multiple infusions of treated cell populations can be made at any of these times.

The non-proliferating, immune functional leukocytes are especially invaluable for DLI in patients for whom traditional allogeneic BMT would be contraindicated, because of advanced age or comorbid disease. Elderly patients, (> 65 years old) presenting cancer are not prime candidates for allo BMT partly because the transplant cannot reconstitute an aged immune system very well and because of the severity of the procedure. Currently, the only option available to control cancer in elderly patients is chemotherapy, the side effects of which can be intolerable for these patients. The treated leukocytes of the present invention provide a much needed alternative to treating cancer in elderly patients and to providing immune defense against opportunistic infections. DLI will be useful to destroy cancerous cells without attendant GVHD.

In certain cases of treatment, it may be desirable to expand subpopulations of the donor leukocytes, particularly antigen-specific T cells and precursor cells, before subjecting the donor leukocyte population to treatment with the above-described compounds. The leukocyte population can be stimulated with an antigen specific to the diseased cell (e.g., tumor-associated antigens) or pathogen to expand/enrich for the number of cytotoxic and helper T cells specific to the antigen. The stimulation can be performed in vivo by vaccination of the donor with the appropriate antigen prior to isolation of the leukocytes from the stimulated donor; alternatively stimulation of isolated leukocytes can be carried out in vitro prior to treatment of the leukocyte population with the compound.

For example, donors vaccinated with IgG from multiple myeloma patients have high levels of myeloma-specific precursor CTLs. Thus, for use in treating multiple myeloma (MM), it is preferable to prepare the leukocytes from such vaccinated donors. Pulsing dendritic cells with tumor-associated peptides for immunization of patients with non-Hodgkin's lymphoma is described in Hsu, FJ et al. Nat. Med. 2:52-58, 1996. Techniques of pulsing dendritic cells with peptide/antigens are reviewed in Stingl, G. et al. Immunol. Today 16:330-333, 1995. cDNA immunization (transfecting the dendritic cell with the appropriate cDNA) is described in Pardoll, D. et al. Immunity 3:165-169, 1995. In adoptive immunotherapy for the treatment of CML, donors can be vaccinated with bcr-abl peptides before isolation of donor leukocytes for treatment by the method of the invention and subsequent infusion. Effective and immunogenic peptides for vaccination are described, e.g., by ten Bosch, G. et al. Blood 88:3522-3527, 1996. In yet another example, donor leukocytes for the treatment of prostate cancer are pre-stimulated with prostate specific antigen (PSA) either in vivo or in vitro, before treatment according to the

method of the invention. Such vaccinations can be applied to boost precursor T cell populations and/or populations of T cells specific to other tumor specific antigens, viral antigens and antigens of other pathogens.

Leukocytes from donors vaccinated with the appropriate antigens are useful prophylactically or therapeutically, for providing an immunocompromised patient with immune defense against opportunistic infections such as cytomegalovirus (CMV), Epstein Barr virus (EBV), Kaposi's Sarcoma associated Herpes virus or adenovirus (Ad) infections. As an example, donors can be vaccinated against CMV antigens and their leukocytes isolated and subjected to treatment according to the methods of the invention for use in DLI to treat patients presenting CMV infections as a result of immunosuppression. Immunocompromised patients include patients under immunosuppressive drug treatment such as organ transplant patients (BM and other organs), HIV or EBV infected individuals, cancer patients, and individuals with immune deficiency diseases.

Donor leukocytes can also be stimulated *in vitro* (also referred to as *ex vivo* expansion) to expand precursor pools. For example, in the case of multiple myeloma, donor leukocytes can be stimulated by *in vitro* culture with patient specific MM antigen presented by dendritic cells, before treatment to block GVHD activity.

*Ex vivo* or *in vitro* stimulation can also be employed to expand the population of antileukemic T cells for the treatment of CML. In CML, the classical translocation of the *c-abl* oncogene on chromosome 9 to the break point cluster region (*bcr*) on chromosome 22 (*t*(9;22)(q34;q11) results in a *bcr-abl* fusion gene and the expression of the *bcr-abl* 210 kD fusion oncoproteins. The *bcr-abl* fusion protein is CML specific and the two main variants of the *bcr-abl* protein are well characterized antigens. *In vitro* stimulation to expand T cells specific for the *bcr-abl* protein can be performed as described, e.g., by Choudhury, A. et al. Blood 89: 1133-1142, 1997; ten Bosch, G. et al. Blood 88:3522-3527, 1996; and Mannering et al. Blood 90:290-297, 1997. Peptides representing the breakpoint region or junction (novel junctional amino acid sequence) can serve as the immunogens for *in vitro* immunization of human T cells from a healthy donor (see ten Bosch et al., 1996, *supra*; Mannering et al., 1997, *supra*). The resultant CD4<sup>+</sup> T cells recognized *bcr-abl* expressing cells from an allogeneic CML patient. In another approach, dendritic cells (DC) can be generated *in vitro* from peripheral blood cells of CML patients and used as antigen-presenting cells for the *ex vivo* expansion of antileukemic T cells

(Choudhury et al., 1997, *supra*). More generally, donor antigen-presenting cells (APCs) can be pulsed with antigen characteristic of a diseased cell, or can be co-cultured with diseased cells (*e.g.*, inactivated tumor cells); and these APCs can then be exposed to donor leukocytes prior to infusion of the donor leukocytes into a recipient. Alternatively, direct  
5 contact between the donor leukocyte and a diseased cell (or a diseased cell antigen) can be used to expand the T-cell population in the donor lymphocytes.

The "antigen" used to expand the T cells can be a polypeptide, a lipid or a carbohydrate moiety (or any combination, *e.g.*, a glycoprotein), and can be isolated from the diseased cell or pathogen or recombinantly produced. A polypeptide-containing  
10 antigen need not constitute a full-length protein, as long as it includes at least one epitope. The antigen can be contained in a vaccine composition as a full length protein or fragment thereof or as a recombinantly produced fusion protein, and may or may not be conjugated or otherwise presented with an adjuvant. The vaccine can take various forms: diseased cell expressing the antigen, or cell membrane preparation thereof; or the pathogen, preferably  
15 in inactivated form. Methods of preparing vaccines are taught in the literature, see, *e.g.*, Harlow, *supra*. Methods of immunization and in vitro (*ex vivo*) stimulation and expansion of precursor cell populations are known in the art, see, *e.g.* Choudhury, A. et al. Blood 89: 1133-1142, 1997; ten Bosch, G. et al. Blood 88:3522-3527, 1996; Mannering et al. Blood 90:290-297, 1997.

For in vivo use, the leukocytes will generally be administered in plasma, synthetic media or other physiologically buffered solution at a dosage of about  $10^5$  to  $10^{11}$   
20 leukocytes per kg weight per infusion, in a volume of about 50 to 500 ml or more. These parameters will vary with the treatment and disease and will be decided by the physicians treating the patient. The standard DLI practice is to administer leukocytes at a dose of  $10^7$   
25 to  $10^8$  cells per kg of body weight. DLI can be administered in conjunction with chemotherapy.

For in vivo uses, the leukocytes of the present invention will generally be administered intravenously.

GVHD is usually apparent within 90 days after the BMT. For alleviating disease in  
30 cancer relapse patients, DLI is performed preferably within 1 week to 3 months after diagnosis of relapse. For adoptive immunotherapy after allo BMT, DLI can be delivered before to several years after BMT, as maintenance therapy. For cancer patients receiving DLI instead of BMT, the DLI is preferably administered soon after diagnosis of the cancer.

In the absence of BMT, DLI can be provided before, during or after chemo and irradiation regimens or other therapeutic regimens. In all indications, the appropriate timing of the DLI will be determined by the physicians of skill in the treatment of the disease.

Assessment of treatment success: After a DLI, the host or patient is monitored for GVHD following routine procedures. GVHD is usually apparent within 90 days post infusion. Clinical GVHD symptoms include skin rash, swelling and lesions in the liver, gut, lungs and joints, severe diarrhea and jaundice. The level of the patient's bilirubin and liver function enzymes can be measured and a liver biopsy can also be done. Patients receiving DLI can be monitored regularly for detailed clinical history, physical examination, general laboratory evaluation including complete blood count and differential, urinalysis, blood urea nitrogen creatinine, bilirubin, aspartate transaminase (AST), alanine transferase (ALT), alkaline phosphatase,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , albumin, total protein, glucose, and radiographic examination. The status of the cancer can be assessed for example, by examination of marrow aspirates and biopsy, cytogenetic examination (including immunohistochemical analysis), and molecular analysis.

For the purposes of this invention, a recipient of the present treated leukocytes is alleviated of the disease (disease refers to malignancy or infection) if there is visible or measurable improvement in the symptoms of or resulting from the disease. The symptoms and methods of assessing improvement in them will vary with the disease condition but will be familiar to the clinician. One useful endpoint for evaluating treatment success is 100 day mortality.

The methods and compositions of the invention are also useful for prevention of GVHD in situations other than DLI. These include, but are not limited to, platelet transfusions and febrile, non-hemolytic transfusion reactions resulting from cytokine accumulation during platelet storage.

The present methods and the treated, non-proliferating leukocytes of the present invention also have in vitro uses. The PCT procedure, for example, is a minimally invasive method of inhibiting DNA synthesis without affecting the biosynthesis of cells in primary cultures or cell lines which have the ability to differentiate, such as progenitor cells and stem cells. Thus, treatment conditions can be used to prepare isolated stem cells, leukocytes or other cell lines in an assay to test whether a cell differentiation or development step is dependent on proliferation/DNA synthesis. Such studies may identify target molecules along the differentiation or development pathway and facilitate

development of drugs for either inhibiting or promoting differentiation. Treated leukocytes can also be used, e.g., as stimulator cells in a one way MLR assay, to stimulate immune responses of proliferation competent allogeneic cells without the simultaneous proliferation of the stimulator cells themselves. PCT and related treatments, using compounds capable of forming a covalent bond with DNA, would replace the current use of irradiation or mitomycin C to achieve cytostasis of the stimulator cells. For these in vitro uses, compositions of treated leukocytes or other cells can be provided either alone or as part of an assay kit. The assay kit can be for MLR or for the differentiation assay. For these purposes, the treated cells will generally be provided in frozen form. Alternatively, kits may provide reagents and instructions for preparation of leukocytes or other cells having the above-described characteristics. The reagents will include one or more compounds capable of forming a covalent bond with DNA. For example, a kit to be used for PCT will include one or more photoactivatable compounds, preferably a PAP or an acridine, more preferably, S-59.

The following examples are intended to illustrate the invention described herein but not to limit its scope. Certain modifications to the method will be readily apparent to one of skill in the art and are encompassed by the claims.

## EXAMPLES

### Various assays

#### Proliferation assay by $^3\text{H}$ Thymidine incorporation.

In a typical assay, cells are grown in 96-well plates for a desired period of time in the presence or absence of the test compound (e.g. DNA replication inhibitor or mitogen). Media is prepared that typically contains 1  $\mu\text{Ci}/50 \mu\text{l}/\text{well}$  using  $^3\text{H}$  thymidine of approximately 20 Ci/mmol specific activity. This media/label is added to the cells and the cells are incubated at 37°C for approximately 4-6 hours or longer. For convenience, a multichannel cell harvester is used to transfer cells to paper filter discs and to wash away free (unincorporated) label. The discs are then dried, transferred to vials and immersed in scintillation fluid. The vials can then be placed in an automated counter, counting for the full tritium range, and the average counts per minute (cpm) for triplicate samples is determined.



### Measurement of compound-DNA adducts

<sup>3</sup>H-labeled PAP was used to measure the extent of DNA modification. PAP was added to the desired final concentrations in the purified leukocyte samples. Aliquots of 20 mL in mini-PL2410 plastic containers were illuminated with the appropriate UV light dose. The control sample was treated with PAP alone without UVA. After illumination, leukocyte DNA was purified. The DNA content of each sample was determined by measurement of absorbance at 260 nm. The number of psoralen adducts per 1,000 base pairs (bp) was calculated from the radioactivity in the DNA samples. A standard curve correlates the amount of <sup>3</sup>H counts with the number of adducts.

### PCR Inhibition Assay

Polymerase chain reaction (PCR) methodology is well known in the art. See K.B. Mullis *et al.*, US Patents Nos. 4,683,195 and 4,683,202, hereby incorporated by reference. This assay can be used to evaluate the effect of PAP-DNA adducts on the template function of specific nucleic acid sequences.

DNA samples (1 µg) obtained from photochemically-treated or gamma irradiated platelet concentrates were PCR amplified for a 242 bp sequence in the HLA-DQα locus or for a 439 bp sequence in the β-globin gene locus. Control (untreated) DNA was serially diluted (1:10) and then amplified. The treated DNA was amplified without dilution. PCR amplification was carried out to 35 cycles. This assay provided a means to correlate functional T-cell proliferation inhibition, as measured in a cell proliferation assay, with direct modification of nucleic acid.

The following examples 1 to 4 demonstrated that the effect of PCT on the properties of treated leukocytes correlates with leukocyte DNA modification and inhibition of polymerase activity. Secondly, PCT treatment of leukocytes in platelet concentrates can prevent transfusion activated GVHD in a murine transfusion model.

### Example 1

#### **Photochemical Inactivation of T-cells**

Example 1 demonstrated that leukocytes can be inactivated by PCT in a dose dependent manner. As exemplary psoralens, S-59, AMT and 8-MOP were used. The PCT

dose dependence varies with the properties of the psoralen used, S-59 being the most efficient tested.

The dose related effect of photochemical treatment with S-59 (Squares), 4'-aminomethyl 4,5',8-trimethylpsoralen [AMT] (triangles), and 8-methoxypsoralen [8-MOP] (circles) on leukocytes in platelet concentrates (PC) was characterized and the results shown in Fig. 1. Platelet concentrates were prepared and treated as described in U.S. Patent No. 5,593,823, incorporated herein by reference. Leukocytes from photochemically-treated and untreated pooled random donor PC were plated in an LDA assay. Varying concentrations of the three psoralens were used with a constant light dose of 1 Joule/cm<sup>2</sup> UVA. T-cells were inactivated to the limit of detection by LDA (indicated by arrows) with 0.05  $\mu$ M S-59, 1.0  $\mu$ M AMT, and 10.0  $\mu$ M 8-MOP. These data clearly demonstrate that S-59 is a superior photoreagent, compared to AMT and 8-MOP, in inhibiting proliferation of T cells.

## Example 2

### **Mixed Leukocyte Reaction**

Peripheral blood was drawn into anticoagulant citrate dextrose (ACD) tubes from four individuals. The peripheral blood mononuclear cells (PBMC's) were isolated by density gradient centrifugation over Ficoll. The PBMC's from three donors were pooled and served as allostimulators. The PBMC's from the remaining individual was used as the responder. The stimulator and responder PBMC's were washed two times with RPMI supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin and resuspended in the supplemented media.

The stimulator PBMC's were exposed to 2500 cGy gamma radiation from a blood bank <sup>137</sup>cesium irradiator. The stimulator cells were then plated in 96 well round bottom plates in aliquots of 100  $\mu$ L containing  $1.0 \times 10^5$  cells.

The responder cells were subjected to PCT with S-59 as follows. Responder cells were resuspended in 30 mL of the above cell culture media and aqueous S-59 stock solution (dissolved in water) was added to the cell suspension to the final concentrations specified in Figure 2, Figure 3 or Figure 4. The concentration of S-59 stock solution and volume used varied depending on the final S-59 concentration desired. The 30 mL cell solution containing S-59 was then transferred to a small PL2410 bag and illuminated with UVA light at a dosage of 3 J/cm<sup>2</sup>. For Figure 3, the light dosage was 3.0 J/cm<sup>2</sup>. The cells

were then centrifuged, resuspended in cell culture media and plated in aliquots of 100  $\mu$ L containing  $1.0 \times 10^5$  cells and mixed with the stimulator cells for a final volume of 200  $\mu$ L per well.

The plates were then incubated at 37°C with 5% CO<sub>2</sub>. Samples of the supernatant from the MLR were removed after 1, 2, and 7 days of incubation and stored at -80°C for subsequent cytokine analysis. Wells that were designated for the measurement of cell proliferation were incubated for 6 days. After the 6 day incubation all wells used to measure cell proliferation received 1  $\mu$ Ci of <sup>3</sup>H thymidine. After 24 hours of incubation in the presence of <sup>3</sup>H thymidine the cells were harvested onto glass fiber paper. The amount of incorporated <sup>3</sup>H thymidine was then quantified by liquid scintillation counting. The results of the <sup>3</sup>H thymidine incorporation by responder cells photochemically treated with various concentrations of S-59 are shown in Figure 2.

Analysis of synthesis of the cytokines IL-2 and IFN- $\gamma$  by treated cells was done with ELISA assay kits provided by R&D systems, according to the manufacturer's instructions. Figures 3 and 4 show the results of the IL-2 and interferon- $\gamma$  production, respectively. In Figure 4, IFN- $\gamma$  analysis was done on the cell culture media taken on day seven.

### **Example 3**

#### **Leukocyte Function Modulation by PCT**

Example 3 demonstrated that leukocyte activity, as measured by cytokine synthesis and surface marker expression (exemplified by CD69 lymphocyte activation marker expression) can be modulated by the concentration of the psoralen and the light dose used for the PCT. The data provide evidence that UVA light doses and concentrations of photoactivatable compounds can be titrated to obtain appropriate ranges effective both to block proliferation of T cells in the leukocyte population, and to maintain leukocyte activity, as demonstrated by cytokine synthesis and surface marker expression.

##### **a. Modulation of Cytokine Synthesis by PCT**

IL-8 synthesis was also measured after PCT with different concentrations of S-59 at a constant light dose of 0.5 J/cm<sup>2</sup> UVA (see Figure 5). It was found that IL-8 synthesis could be modulated by PCT with increasing concentrations of S-59. A similar behavior

was obtained at 1.0 J/cm<sup>2</sup> UVA over a range of S-59 concentrations (data not shown). These results demonstrate that cytokine synthesis can be modulated by the concentration of the psoralen and dose of the light used for PCT.

**b. Modulation of CD 69 expression on leukocytes after PCT treatment**

The induction of the CD69 expression by leukocytes upon their activation by PMA and calcium ionophore was measured with the use of a fluorescent antibody to CD69 and FACS-Scan analysis (see Figure 6). The expression of CD69 was measured as a function of time after photochemical treatment with different concentrations of AMT and 1 J/cm<sup>2</sup> UVA. The effect of PCT on the induction of CD69 expression was also found to be psoralen dose dependent, with expression of CD69 showing greater inhibition at increasing concentrations of AMT. At the low doses anticipated for S-59 in this study, induction will remain intact.

**Example 4**

**DNA modification by PCT**

Example 4 demonstrated that the effect of PCT on the properties of the treated leukocytes correlates with leukocyte DNA modification and inhibition of polymerase activity.

Psoralen adducts on leukocyte genomic DNA were measured after photochemical treatment in pooled random donor PC by using <sup>3</sup>H radiolabeled S-59, AMT, and 8-MOP plus 1.9 J/cm<sup>2</sup> UVA and scintillation counting of the isolated DNA of treated leukocytes (Figure 7). Photochemical treatment with 150 μM S-59, AMT, and 8-MOP induced 12.0, 6.0, and 0.7 adducts/1000 bp of DNA respectively. The relative number of adducts after PCT on the genomic DNA of leukocytes treated correlated with the effect that PCT had on the leukocyte functions and demonstrated that the effect was a direct consequence of the DNA modification. Inhibition of DNA amplification by DNA polymerase (data not shown) in a PCR-inhibition assay showed the same dependence on the concentration of the psoralen used during PCT.

The results from all of the biological and molecular parameters used here to study the effects of PCT on leukocytes *in vitro* are consistent. Assessment of leukocyte function, by limiting dilution analysis, measurement of cytokine production, and quantitation of

psoralen induced DNA damage showed that PCT inactivates leukocytes in a dose dependent manner. Using appropriate doses, functions of the leukocytes such as the production of cytokines or the expression of antigenic markers can be controlled. Additionally, of the three psoralens tested S-59 is the most efficient in inactivating leukocytes. The dose range within which S-59 inhibits T-cell proliferation is approximately 3.5 log units. Therefore, the dose window within which leukocyte proliferation and attendant GVHD can be inhibited but immune function is retained is larger than that provided by other means of inactivation.

### Example 5

#### **Prevention of TA-GVHD in a murine transfusion model**

The following murine transfusion model is a well characterized model that simulates the human clinical syndrome of TA-GVHD (Fast, LD et al. Blood 82: 292, 1993). Based on the promising *in vitro* results, the effect of S-59 photochemical treatment on the inhibition of TA-GVHD was evaluated, using this *in vivo* model, by transfusing splenocytes from a homozygous parent (strain A, H-2<sup>a</sup>) into immunocompetent heterozygous F<sub>1</sub> hybrid recipients (strain B6AF<sub>1</sub>, H-2<sup>a/b</sup>).

Each recipient (F<sub>1</sub>) was transfused with approximately 10<sup>8</sup> donor (A or F<sub>1</sub>) splenocytes via the lateral tail vein. Splenocytes obtained from density gradient centrifugation were used as the source of viable T cells. Three transfusion groups were utilized: (1) handling control group (F<sub>1</sub>→F<sub>1</sub>): B6AF<sub>1</sub> splenocytes were transfused into B6AF<sub>1</sub> recipients; (2) positive TA-GVHD control group (A→F<sub>1</sub>): donor A splenocytes were transfused into B6AF<sub>1</sub> recipients; (3) S-59 photochemical treatment group (PCT A→F<sub>1</sub>): donor A splenocytes were treated with 150 μM S-59 and 3 Joules/cm<sup>2</sup> UVA and then transfused into B6AF<sub>1</sub> recipients.

Recipients were monitored for biological evidence of TA-GVHD two weeks post-transfusion. Spleens of recipients were analyzed for donor T-cell engraftment using two-color, fluorescence-activated flow cytometry. In this assay, splenic T-cells were stained with a pan-T-cell, anti-CD3 antibody and also with an anti-H-2<sup>b</sup> antibody. Donor A T-cells were detected by the absence of reaction with the anti H-2<sup>b</sup> antibody. In control experiments, greater than 99% of CD3 positive spleen cells from F<sub>1</sub>→F<sub>1</sub> recipients were labeled with the anti-H-2<sup>b</sup> antibody. In parallel, the presence of cytotoxic lymphocytes

(CTLs) in the spleens of recipients was measured by using a  $^{51}\text{Cr}$ -lysis assay. Spleen cells were incubated with  $^{51}\text{Cr}$ -labeled EL-4 cells (H-2<sup>b</sup>) at an effector: target ratio of 150:1 for 4–6 hours at 37°C. The cytotoxic activity of CTLs was determined by the target cell lysis and release of  $^{51}\text{Cr}$  into the culture medium.

5           The results indicated that while recipients in the control  $F_1 \rightarrow F_1$  group remained healthy throughout the duration of the study, recipients in the  $A \rightarrow F_1$  group developed biological signs of TA-GVHD two to three weeks after transfusion of donor spleen cells. TA-GVHD was characterized by splenomegaly, donor T-cell (H-2<sup>a</sup>) engraftment ( $28.6 \pm 11.3\%$ ) and the presence of CTLs ( $35 \pm 18.7\%$   $^{51}\text{Cr}$  lysis) in recipient spleens  
10 (Table 1).

Splenomegaly was evaluated two weeks post-transfusion by measuring the spleen: body weight ratio. Development of immunodeficiency was detected by evaluation of thymic cellularity 3 weeks post-transfusion. Thymic hypoplasia is a reliable index of acquired immune deficiency associated with TA-GVHD.

15           Recipients were also monitored for clinical symptoms of GVHD which included body weight, posture, activity, skin integrity, fur texture, white blood cell count, red blood cell count, and platelet count. Weekly body weight of the healthy animals in the  $F_1 \rightarrow F_1$  group increased over time while the average body weight of animals in the  $A \rightarrow F_1$  group failed to increase over time

20           The average clinical score (grade 0 to 2, healthy animals were graded 0) of animals in the  $A \rightarrow F_1$  group increased over time indicating that TA-GVHD was outwardly apparent and progressively more severe. Animals in the PCT  $A \rightarrow F_1$  group were similar to animals in the control  $F_1 \rightarrow F_1$  group and remained healthy and free of visible clinical manifestation of TA-GVHD. Twenty-five percent mortality was observed in the  $A \rightarrow F_1$  group when  
25 followed for more than 10 weeks post-transfusion, whereas all animals in the control  $F_1 \rightarrow F_1$  and PCT  $A \rightarrow F_1$  groups remained healthy.

TABLE 1

Biological Evaluation and Cell Counts of Transfused B6AF <sub>1</sub> Recipients (N = 5 to 11)			
Parameter	F <sub>1</sub> →F <sub>1</sub> (Mean ± S.D.) <sup>1</sup>	A→F <sub>1</sub> (Mean ± S.D.)	PCT A→F <sub>1</sub> (Mean ± S.D.)
% Donor A T-cells <sup>2</sup>	1.1±0.7	28.6±11.3	1.0±0.7
% Lysis ( <sup>51</sup> Cr) <sup>2</sup>	11.5±3.3	35±18.7	18.0±4.2
Spleen wt/body wt (x10 <sup>3</sup> ) <sup>2</sup>	3.6±0.4	25.9±1.8	2.7±0.4
Thymocyte count (x10 <sup>6</sup> ) <sup>3</sup>	27.0±11.0	7.5±2.9	27±4.0
WBC count (10 <sup>3</sup> /μl) <sup>3</sup>	7.6±2.8	2.5±0.5	7.1±1.5
RBC count (10 <sup>6</sup> /μl) <sup>3</sup>	8.8±0.3	7.4±0.8	8.6±0.2
Platelets (10 <sup>3</sup> /μl) <sup>2</sup>	1248±201	1038±167	1291±159

1. S.D. = standard deviation

2. Measured two weeks post-transfusion

3. Measured three weeks post transfusion

Tissue sections were prepared and examined for histologic abnormality after blind coding. The liver was evaluated for the presence of lymphoid infiltrates with bile duct destruction and vascular endothelial inflammation and infiltration. Splenic histology was assessed for preservation or destruction of lymphoid follicles. Skin sections were evaluated for lymphoid infiltrates in sub-dermal areas and appendages. Bone marrow sections were evaluated for cellularity and maturation within each major lineage: myeloid, erythroid, and megakaryocytic. A→F<sub>1</sub> mice developed histologic evidence of GVHD in liver, spleen, bone marrow, and oral mucosa in a blinded study. In contrast, recipients of either photochemically treated donor cells (PCT-A) or syngeneic cells (F<sub>1</sub>) showed no histologic abnormalities.

Transfusions for groups of mice where the infused cells were treated with S-59 concentrations varying from 50 nM to 150 μM and 3 J/cm<sup>2</sup> UVA, showed complete GVHD inhibition over this range. This demonstrates that the dose window within which GVHD can be inhibited by PCT *in vivo* is similar to the one obtained for the *in vitro* assays.

In conclusion of the *in vitro* and *in vivo* data, PCT treatment of leukocytes can inhibit their proliferation *in vitro* and inhibit TA-GVHD *in vivo* over 3.5 log units of S-59 concentration. For the same range of concentrations, a dose-dependent modulation of the

leukocyte functions *in vitro* (cytokine synthesis and surface molecule expression) was observed.

### Example 6

5           This example describes the approach for determining the PCT conditions suitable to suppress GVHD while preserving the GVL effect of DLIs for application, e.g., in the treatment of relapsing leukemia patients post-allogeneic BMT. The experimental objectives are summarized below.

- 10           A. Determination of the psoralen concentration range to be used in PCT so as to prevent proliferation of lymphocytes *in vitro* and thus inhibit GVHD.
- B. Characterization of the phenotype of photochemically treated leukocytes *in vitro* to determine cell viability and expression of T and NK cell surface antigens indicative of generating a GVL effect *in vivo*.
- 15           C. Qualitative and quantitative analysis of cytokine expression by treated leukocytes *in vitro*.
- D. Demonstration of inhibition of GVHD by treated leukocyte-DLIs *in vivo* on a murine leukemia model.

### Photochemical treatment of leukocytes with psoralens

20           Stock solutions of psoralen are prepared as exemplified below. A 15 mM stock solution of AMT was prepared by dissolving 50 mg of AMT powder in 10 mL of distilled water. The solution was mixed vigorously and filtered through a 0.2  $\mu$ m syringe filter. The concentration of AMT in the filtered solution was determined by measuring the  
25           absorbance of the solution at 250 nm using a Shimadzu UVI60U spectrophotometer. The AMT concentration was calculated using a value of 25000 M<sup>-1</sup>cm<sup>-1</sup> for the extinction coefficient.

30           A stock solution of S-59 psoralen was prepared by dissolving a powder of S-59 in distilled water. The solution was mixed vigorously and filtered through a 0.2  $\mu$ m syringe filter. The concentration of S-59 in the filtered solution was determined by measuring the absorbance of the solution at 250 nm using a Shimadzu UV160U spectrophotometer. The S-59 concentration was calculated using a value of 25400 M<sup>-1</sup>cm<sup>-1</sup> for the extinction coefficient.



8-methoxypsoralen (8-MOP) is poorly soluble in aqueous solutions. Therefore, leukocyte preparations that were treated with 8-MOP and ultraviolet light were prepared using PAS solution (PAS, available from Baxter, is an artificial blood substitute consisting of sodium acetate, trisodium citrate sodium chloride phosphate buffer pH=7.2 at 300 mOsm) saturated with 8-MOP. The saturated solution was prepared by suspending 100 mg of 8-MOP in 100 mL of PAS. The solution was agitated for 16 hours at room temperature in a dark container. The resulting solution was filtered through a 0.2  $\mu$ m filter to remove any undissolved 8-MOP. The final 8-MOP concentration was determined by measuring absorbance at 248 nm using a Shimadzu UV160U spectrophotometer. The 8-MOP concentration was calculated using a value of 22900 M<sup>-1</sup>cm<sup>-1</sup> for the extinction coefficient.

The stock psoralen solutions are added to suspensions of purified donor leukocyte populations to the desired final concentration. Units that were treated with 8-MOP were prepared by using PAS saturated with 8-MOP in the preparation procedure discussed above. The purified donor leukocytes are illuminated with UVA light to a final dose of between 10<sup>-3</sup> to 100 J/cm<sup>2</sup> in a photoactivation device. The units were agitated at 70 cycles/min during illumination. Samples of the leukocytes are taken prior to illumination for use as controls.

The S-59 is added to the purified leukocyte preparation at a concentration of between 10<sup>-4</sup> and 150  $\mu$ M. The leukocytes will be provided at a cell density of 10 to 10<sup>8</sup> cells/mL, preferably at 10<sup>7</sup> cells/mL, more preferably at 2 x 10<sup>6</sup> cells/mL.

The sample of a leukocyte population mixed with S-59 is exposed to ultraviolet light of wavelength of between 200 and 450 nm, preferably 320-400 nm. The cells will preferably receive a light dose in the range of about 10<sup>-3</sup> to 100 J/cm<sup>2</sup>. In a preferred embodiment, the light dose is at 3 J/cm<sup>2</sup>. The time period for UV light exposure will be about 1 second to 60 minutes, preferably about 1 minute.

After PCT, the leukocyte samples will be assayed for the ability to proliferate and to participate in leukocyte activity, as described above and in the Examples. The following will be measured: cell viability and integrity; proliferation activity; surface antigen expression; cytokine secretion; GVHD and GVL activity.

The following assays can be applied to both alkylator compound-treated or photochemical treated leukocytes.

A. Dose Response Kinetics of Leukocytes *in vitro*i). Limiting Dilution Assay (LDA)

This assay directly measures the number of clonable T cells, which correlates directly *in vivo* with the incidence and severity of GVHD (Kernan, N.A. *et al.*, Blood 68:770–773, 1986). LDA was used by the FDA to set the current guideline for gamma irradiation of blood products. This assay is conducted according to the procedure of Pelsynski, M. *et al.*, Blood 83:1683–1689, 1994. Briefly, human leukocytes are assayed for the number of proliferating cells after PCT treatment with different concentrations of drug and a constant dose of light. Lethally  $\gamma$ -irradiated pooled allostimulator cells are used to stimulate growth. Controls for proliferation include untreated leukocytes (as positive control for proliferation) and pre-PCT leukocytes that have been lethally  $\gamma$ -irradiated (negative control). Growth is measured by the number of isolated proliferating colonies. A correlation between the dose used and the number of cells that are proliferation-inhibited is determined for human leukocytes. The dose response obtained from this experiment is expected to be similar to the one obtained for the leukocytes in platelet concentrates.

LDA was performed as follows. Leukocytes were isolated from peripheral blood by leukapheresis. Viable T-cells were stimulated to proliferate in wells of microtiter plates containing RPMI medium supplemented with fetal bovine serum (FBS), phytohemagglutinin (PHA), recombinant interleukin-2 (rIL-2) and T-cell growth factor (TCGF). Each well also contained  $10^5$  allostimulator cells prepared from a pool of PBMCs from ten individuals and irradiated with 5000 cGy of gamma irradiation.

Leukocytes from each untreated control sample were diluted in two independent series. Aliquots of 100  $\mu$ L containing in one series  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ ,  $10^1$  and  $10^0$  cells or in a second series 300, 100, 33, 11 and 4 cells were plated per well. Each dilution was plated in ten replicates. An 11 mL aliquot of the photochemically treated sample containing  $1.1 \times 10^7$  total leukocytes was cultured to detect viable T-cells. Aliquots of 100  $\mu$ L from this sample were plated undiluted into 110 wells.

The microtiter plates were incubated in a CO<sub>2</sub> incubator at 37°C for 3 weeks. After 0.5, 1, and 2 weeks of incubation, each well was fed with additional TCGF, FBS, rIL-2 and PHA. At the end of the 3-week incubation period, each well was scored for the presence of T-cell clones. Wells with one or more T-cell clones were scored positive. Wells with no T-cell clones were scored negative. The T-cell frequency for each sample was calculated using a minimum chi square analysis based on the Poisson distribution.

The T-cell reduction factor was calculated using the formula: T-cell reduction factor =  $f_{\text{control}}/f_{\text{treated}}$ , where  $f_{\text{control}}$  and  $f_{\text{treated}}$  are T-cell frequencies for the control and treated samples, respectively.

Control aliquots were either not treated or were treated with UVA only or S-59 only. One aliquot was irradiated with the clinical dose of 2500 cGy of gamma. To the remaining aliquots, S-59 was added to final concentrations ranging from  $10^{-4}$   $\mu\text{M}$  to 150  $\mu\text{M}$ . They were then illuminated at a UV light dose ranging from  $10^{-3}$  to 100 Joules/cm<sup>2</sup>.

ii). Mixed Lymphocyte Reaction (MLR) Assay

This assay, also known as the MLC test is conducted according to the procedure of Kraemer, K.H. *et al.*, J. Inv. Derm. 77:235–239, 1981 and Kraemer, K.H. *et al.*, J. Inv. Derm. 76:80–87, 1981. For a review of the rationale and controls in this assay, see Basic and Clinical Immunology, 8th edition, Daniel P. Stites, Abba I. Terr and Tristram G. Parslow (eds.), Appleton & Lange, Norwalk, CT, 1994, pp. 246-247. It indirectly measures the DNA synthesis induced in lymphocytes by allostimulation. When the lymphocytes of 2 HLA-disparate individuals are combined in tissue culture, the cells enlarge, synthesize DNA, and proliferate, whereas HLA-identical cells remain quiescent. The DNA synthesis induced is determined through the incorporation of <sup>3</sup>H-thymidine into the nucleic acid inside the proliferating cells. Cells are then harvested, washed free of unbound radioactivity and counted in a beta counter for internalized radioactivity.

The leukocytes are assayed for their responder and stimulator functions in one-way MLR. The standard, appropriate positive and negative controls are included. The responder function is assayed by incubating responder cells with lethally  $\gamma$ -irradiated pooled human leukocytes (stimulator cells). The stimulator function of the treated leukocytes (treated by PCT or other alkylator compounds) is determined, after they have been lethally  $\gamma$ -irradiated to block thymidine uptake, by incubating with normal pooled human leukocytes. The results obtained as a function of treatment dose are compared to a two-way MLR.

Although the MLR will provide a subset of the information provided by LDA, it is able to provide information on the ability of treated leukocytes to stimulate other cells. Stimulation by treated leukocytes in an MLR can be used as a model for the induction of an immune response against leukemia, activated by the DLI in a BMT transfused patient.

In that case, the immune response against leukemia (GVL effect) may be launched by the host or donor cells present in the BMT.

B. Phenotypic Characterization of Treated Leukocytes

5 i). Surface Antigenic Marker Expression

The effect of PCT or alkylator treatment on expression of surface antigenic markers is determined through the use of the appropriate fluorescently labeled antibodies (Pharmingen) and standard FACS-SCAN analysis.. The presence of antigenic markers, CD2, CD28, CTLA4, CD40 ligand (gp39), CD18, CD25, CD69 and CD16/CD56, which  
10 are known to be involved in interactions associated with T-cell and NK cell activation and immune function, will be determined as a function of treatment dose, through the use of the appropriate fluorescently labeled antibodies (Pharmingen) and standard FACS-SCAN analysis as described in Coligan, J.E. *et al.*, Current Protocols in Immunology: Current Protocols. New York, John Wiley & Sons, Inc., 1991. Stability of surface molecules  
15 under the treatment conditions is not expected to be influenced given the mild nature of PCT and related treatments on proteins; the induction of their expression is, however, expected to be dose dependent.

See Example 14 for characterization of treated human lymphocytes with respect to proliferation, surface marker expression, cytokine synthesis and cytotoxic activity.

20 ii). Leukemia Cell Line Lysis

To assay for a direct GVL effect induced by treated cells, the cytolytic ability is investigated against a series of human and mouse leukemia cell lines (available from ATCC). The experiment is conducted according to the conditions of Jiang, Y.Z. *et al.*,  
25 Bone Marrow Transplant. 8:233–238, 1991. Leukemia cells are labeled under standard conditions with  $^{51}\text{Cr}$  as described in Coligan, J.E. *et al.*, Current Protocols in Immunology: Current Protocols. John Wiley & Sons, Inc., New York, 1991. Any lysis achieved will be measured by the release of  $^{51}\text{Cr}$  radioactivity in the supernatant and compared to controls for background and total lysis. The effect of the treatment dose on the cytolytic ability of  
30 treated T-cells is monitored. The experiment is performed by adding increasing amounts of treated leukocytes to see if lysis occurs. The experiment can be repeated with subsets of T-cells. Although direct lysis of the leukemia lines provides a direct mechanism for the GVL effect, lack thereof may imply an indirect mechanism for the induction of the GVL

effect by DLI. Therefore, alternatively, cytotoxic activity can be determined by measuring cytokine production, e.g., IFN- $\gamma$ , IL-2 or GM-CSF production.

iii) Viability of Cells *in Vivo*

5 To assess the viability of treated cells *in vivo*, after treatment at varying doses, male mouse leukocytes are infused in female syngeneic or allogeneic recipients. Presence of the treated cells in circulation is monitored through the use of a PCR procedure (Goodarzi, M.O. *et al.*, Transfusion 35:145–149, 1995) applied on blood samples taken at the desired time points after the splenocyte infusion. This procedure detects the presence of a Y-  
10 chromosome-specific gene sequence present only in the male (donor) cells and has a sensitivity of 1-5 cells per 50  $\mu$ L of host blood. This experiment will determine the effect on the clearance of the infused cells in a living animal as well as the relationship between the treatment dose and cell circulation *in vivo*. The viability of treated cells *in vivo* can also be monitored by PKH-26 labeling of the leukocytes before infusion as described in  
15 Johnson et al. Scand. J. Immunol. 45:511-514, 1997.

C. Quantitative and qualitative characterization of cytokine synthesis

Quantitative and qualitative cytokine measurements are conducted through the use of commercially available Elisa kits (R&D Systems) in culture supernatants following co  
20 cultivation of effector cells with stimulator cells. Assays are performed according to the instructions provided by the Elisa manufacturer. Results are evaluated by comparing absorbance measurements for each sample to a standard curve generated by cytokine standards supplied with each assay kit. Cytokine measurements are performed on treated leukocytes as a function of the compound concentration and, in the case of PCT, of the  
25 light dose, as well as the time interval between treatment and measurement. IL-1, IL-2, IL-4, IL-10, IFN- $\gamma$  cytokines are measured because of their established role in T-cell activation and GVHD/GVL.

Measurement of cytokine synthesis by treated leukocytes is important in determining the capability of the cells to function as inducers of an immune response  
30 through their production; it also serves as a measure of biochemical function and protein synthesis.

See Example 14 for characterization of treated human lymphocytes with respect to proliferation, surface marker expression, cytokine synthesis and cytotoxic activity.

D. Use of an in vivo murine model to demonstrate inhibition of GVHD and induction of a GVL effect

Mice are a well established model for studying graft vs host disease and graft  
5 versus leukemia effects. In order to differentially assess the effect of treated leukocytes on GVHD and GVL, the transplantation murine model described in Johnson, B.D. *et al.*, Bone Marrow Transplant. 11:329–336, 1993, and in Johnson, B.D. *et al.*, Blood 85:3302–3312, 1995, is used. In this model female AKR/J mice (H-2<sup>k</sup>) were sublethally irradiated (9 Gy) and then given 10<sup>7</sup> BM cells plus 3 X 10<sup>7</sup> spleen cells from MHC-matched female B10.BR  
10 donors (H-2<sup>k</sup>). This treatment has been shown to result in acute GVHD clinical symptoms and death within 50 days. Elimination of the splenocytes from the transfusion results in complete survival of mixed chimeras, while no BMT results in 50% mortality. In this Example, mouse splenocytes are subjected to treatment before transfusion to test the inhibition of GVHD by the treatment, as measured by mortality, and also to provide the  
15 lowest dose to inhibit the onset of GVHD. Splenocytes are the accepted substitute for blood leukocytes in mice since the volume of blood in mice is too small for such experiments.

To assess the GVL reactivity of treated cells, 21 days post BMT, the recipients are infused with, for example, PA allogeneic splenocytes and then challenged 7 days later with  
20 AKR leukemia cells. Infusion of the mice with 5 X 10<sup>4</sup> AKR leukemia cells after recovery from BMT (28 days) is known to result in leukemia and 95% mortality 25 days after the leukemia challenge (Johnson, B.D. *et al.*, Transplantation 54:104–112, 1992). On the contrary, infusion with allogeneic splenocytes 21 days after BMT has been shown to induce a GVL effect upon challenge with AKR cells 7 days later. This course of action  
25 results in 70% survival after 70 days. If prevention of GVHD has been demonstrated, treatment of the splenocytes before transfusion will allow the detection of induction of GVL in the absence of GVHD. This is measured by the survival rate of treated splenocyte-infused BMT recipients, which were challenged with AKR cells.

The effect of DLI using treated leukocytes on the survival of a leukemic challenge  
0 is tested as a function of the treatment dose and compared with a control group where infusion of untreated splenocytes is performed. The surviving mice are tested for chimerism and leukemic load through the use of a reported PCR assay as described in Johnson, B.D. *et al.*, Bone Marrow Transplant. 11:329–336, 1993).

In this experimental model, a leukemic cause of death can be diagnosed by either white cell count or PCR analysis of the blood immediately before death, since leukemia will cause an expansion of the AKR leukemic cells. Primers specific to AKR leukemia cell specific markers will be used for the PCR analysis. Death from GVHD is diagnosed by low white cell count and spleen atrophy, as well as through characteristic clinical symptoms (fur and posture changes, sticky feces etc.). The efficacy of PA-DLI against minimal residual disease or higher tumor load is tested by injecting smaller or larger numbers of AKR leukemia cells.

Treatment of leukocytes according to the invention, for example by the PCT approach, is deemed to be efficient to induce a GVL effect if, under conditions which do not cause GVHD, it induces a statistically significant increase in the survival of the mice treated with the treated-cell DLI, after a leukemic challenge is administered. The genetic type of the surviving animals should also be completely chimeric as this has been associated in humans with a higher incidence of a disease-free long-term survival. Increase of survival will be easy to demonstrate within 100 days after the original BMT.

Splenocytes will be subjected to treatment under conditions described above for donor leukocytes.

See Example 15 for provision of GVL effect in the absence of GVHD by treated leukocytes in a murine model system.

### **Examples 7-12**

The following specific examples are presented to illustrate the preparative methods for representative alkylator compounds useful in the method of this invention, to provide relevant data regarding the compounds useful to the practitioner, and to illustrate the manner in which the effectiveness of the compounds is determined. All NMR spectra were recorded on a Varian 200 MHz instrument in CDCl<sub>3</sub> unless otherwise noted; chemical shifts are reported versus tetramethylsilane (TMS). IR spectra were recorded with a Perkin Elmer FTIR. HPLC was carried out with a YMC C8 column in a gradient mode using 5 mM aq. H<sub>3</sub>PO<sub>4</sub> as mobile phase A and 5 mM CH<sub>3</sub>CN as mobile phase B. Samples were prepared in DMSO or ethanol and kept at  $\leq 15$  °C prior to injection.

Table 2 indicates the designation of compound number used for the various compounds.

**Table 2**

COMPOUND NUMBER	CHEMICAL NAME
IV	$\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester
V	$\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester
VI	4-aminobutyric acid N-[(2-carbomethoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester
VII	5-aminovaleric acid N-[(2-carbomethoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester
VIII	$\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 3-[bis(2-chloroethyl)amino]propyl ester
IX	$\beta$ -alanine, N-(4-methoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester
X	$\beta$ -alanine, N-(3-chloro-4-methylacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester
XI	$\beta$ -Alanine, [N,N-bis(2-chloroethyl)], 3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl ester
XII	$\beta$ -Alanine, [N,N-bis(2-chloroethyl)], 2-[(6-chloro-2-methoxyacridin-9-yl)amino]ethyl ester
XIII	$\beta$ -Alanine, N-(6-chloro-2-methoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester
XIV	[N,N-bis(2-chloroethyl)]-2-aminoethyl 4,5',8-trimethyl-4'-psoralenacetate

5

**Example 7**

Synthesis of  $\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride (Compound IV,)

Step A.  $\beta$ -Alanine, N-(*tert*-butoxycarbonyl), 2-[bis(2-hydroxyethyl)amino]ethyl ester

10

To a stirred solution of N-(*tert*-butoxycarbonyl)- $\beta$ -alanine (20.3 g, 107 mmol) and 4-methylmorpholine (13.0 mL, 12.0 g, 119 mmol) in dry THF (200 mL) at -15 °C under N<sub>2</sub> was added isobutyl chloroformate (13.9 mL, 14.6 g, 107 mmol) resulting in the immediate formation



of a white precipitate (4-methylmorpholine•HCl). The reaction mixture was stirred at -15 °C for 5 min. followed by the transfer of the reaction mixture to a flask containing a stirred solution of triethanolamine (48.3 g, 324 mmol) in dry THF (150 mL) at -15 °C. The reaction mixture was allowed to warm to 23 °C and stirred for an additional 1.5 h. followed by removal of the precipitate by vacuum filtration. The THF was then removed *in vacuo* from the filtrate and the remaining viscous yellow oil was partitioned between water (500 mL) and EtOAc (5 x 150 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub>. Removal of solvent *in vacuo* gave 25.8 g (75%) of the desired product, β-Alanine, N-(*tert*-butoxycarbonyl), 2-[bis(2-hydroxyethyl)amino]ethyl ester, as a pale yellow oil. <sup>1</sup>H NMR: δ 5.32 (br s, 1 H), 4.18 (t, J = 5.4 Hz, 2H), 3.58 (t, J = 5.1 Hz, 4 H), 3.37-3.23 (m, 2H), 2.80 (t, J = 5.4 Hz, 2H), 2.69 (t, J = 5.1 Hz, 4 H), 2.51 (t, J = 6.0 Hz, 2 H), 1.41 (s, 9 H) The hydroxyl protons were not observed. <sup>13</sup>C NMR: δ 173.0, 156.4, 79.8, 63.3, 60.2, 57.3, 54.1, 36.7, 35.3, 28.8.

Step B. β-Alanine, N-(*tert*-butoxycarbonyl), 2-[bis(2-*tert*-butyldimethylsilyloxyethyl)amino]ethyl ester

A stirred solution of the β-Alanine, N-(*tert*-butoxycarbonyl), 2-[bis(2-hydroxyethyl)amino]ethyl ester from step A (22.7 g, 70.9 mmol) and imidazole (11.1 g, 163 mmol) in acetonitrile (70 mL) under N<sub>2</sub> was cooled to 0 °C. *Tert*-butyldimethylsilyl chloride (534 mg, 3.54 mmol) was then added and the reaction mixture was stirred for an additional 5 min. at 0 °C. The reaction mixture was allowed to warm to 23 °C and stirred for 2 h followed by removal of the resultant white precipitate (imidazole•HCl) by vacuum filtration. The acetonitrile was removed *in vacuo* from the filtrate and the remaining material was partitioned between saturated brine (600 mL) and EtOAc (3 x 200 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub>. Removal of solvent *in vacuo* gave 35.2 g (90%) of the desired product, β-Alanine, N-(*tert*-butoxycarbonyl), 2-[bis(2-*tert*-butyldimethylsilyloxyethyl)amino]ethyl ester, as a yellow oil. <sup>1</sup>H NMR : δ 5.29 (br s, 1 H), 4.14 (t, J = 6.0 Hz, 2 H), 3.65 (t, J = 6.3 Hz, 4 H), 3.37 (apparent q, 2 H), 2.85 (t, J = 6.0 Hz, 2 H), 2.71 (t, J = 6.3 Hz, 4 H), 2.49 (t, J = 5.9 Hz, 2 H), 1.42 (s, 9 H), 0.88 (s, 18 H), 0.03 (s, 12 H); <sup>13</sup>C NMR: δ 172.7, 156.3, 79.7, 63.3, 62.4, 57.7, 54.3, 36.7, 35.3, 28.9, 26.4, 18.7, -4.9.

Step C. β-Alanine, 2-[bis(2-*tert*-butyldimethylsilyloxyethyl)amino] ethyl ester

To a flask containing  $\beta$ -Alanine, N-(*tert*-butoxycarbonyl), 2-[bis(2-*tert*-butyldimethylsilyloxyethyl)amino]ethyl ester from step B (3.01 g, 5.48 mmol) was added neat trifluoroacetic acid (5 mL) resulting in the evolution of CO<sub>2</sub> gas. The reaction mixture was stirred for 5 min. and the trifluoroacetic acid was removed *in vacuo*. The remaining material was  
5 partitioned between saturated NaHCO<sub>3</sub> (100 mL) and EtOAc (3 x 30 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub>. Removal of solvent *in vacuo* gave 2.45 g (100%) of the desired product,  $\beta$ -Alanine, 2-[bis(2-*tert*-butyldimethylsilyloxyethyl)amino]ethyl ester, as a pale yellow oil. <sup>1</sup>H NMR:  $\delta$  4.12 (t, J = 6.0 Hz, 2 H), 3.63 (t, J = 6.4 Hz, 4 H), 2.96 (t, J = 6.2 Hz, 2 H), 2.84 (t, J = 6.0 Hz, 2 H), 2.69 (t, J = 6.4 Hz, 4 H), 2.44 (t, J = 6.2 Hz, 2 H), 0.86 (s, 18 H), 0.03 (s, 12  
10 H) The amine protons were not observed. <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  173.0, 63.4, 62.6, 57.9, 54.4, 38.4, 38.1, 26.4, 18.7, -4.9.

Step D.  $\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 2-[bis(2-hydroxyethyl)amino]ethyl ester

The  $\beta$ -Alanine, 2-[bis(2-*tert*-butyldimethylsilyloxyethyl)amino]ethyl ester (736 mg, 1.64 mmol) was reacted with methyl 9-methoxyacridine-2-carboxylate (669 mg, 2.50 mmol) by stirring in 10 mL of CHCl<sub>3</sub> for 12.5 h at room temperature. The precipitate (acridone) was then filtered off and the filtrate partitioned between saturated aqueous NaHCO<sub>3</sub> (100 mL) and CHCl<sub>3</sub> (3 x 35 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to give  
15 1.61 g of viscous brown oil. Deprotection of the resultant diol was carried out by dissolving the crude intermediate in 3.0 mL of THF under N<sub>2</sub> and, upon cooling to 0 °C, treating with HF/pyridine (1.0 mL). The solution was allowed to warm to room temperature with stirring for 1 h. The volatiles were removed *in vacuo* and the residue was partitioned between saturated aqueous NaHCO<sub>3</sub> (100 mL) and CHCl<sub>3</sub> (3 x 35 mL). The combined organic layers were dried and  
20 concentrated to give 649 mg of a brownish yellow solid. Preparative TLC (C-18, CH<sub>3</sub>CN) gave a 20 % yield of the desired diol,  $\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 2-[bis(2-hydroxyethyl)amino]ethyl ester (>80% pure by HPLC); <sup>1</sup>H NMR:  $\delta$  8.82 (s, 1 H), 8.21-7.94 (m, 2 H), 7.94-7.72 (m, 2 H), 7.59 (apparent t, 1 H), 7.23 (apparent t, 1 H), 4.30-4.18 (m, 2 H), 4.18-4.05 (m, 2 H), 3.89 (s, 3 H), 3.69-3.50 (m, 4 H), 2.92-2.73 (m, 4 H), 2.73-2.55 (m, 4 H) The  
25 amine and hydroxyl protons were not observed.

Step E.  $\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride

Conversion of  $\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 2-[bis(2-hydroxyethyl)amino]ethyl ester to the dichloro compound was achieved by a method similar to that of Peck, et al. (*J. Am. Chem. Soc.* 1959, 81: 3984). A yellow solution of the product from step D (41 mg, 0.090 mmol) in neat  $\text{SOCl}_2$  (6 mL) was stirred at room temperature for 20 hours. The  $\text{SOCl}_2$  was then removed *in vacuo* to give a yellow solid (dihydrochloride salt). The material was then partitioned between saturated  $\text{NaHCO}_3$  (50 mL) and  $\text{CH}_2\text{Cl}_2$  (3 x 20 mL). The combined organic layers were dried over  $\text{Na}_2\text{SO}_4$ . Removal of solvent *in vacuo* gave 35.4 mg of the dichloro compound free base as an orange gum.  $^1\text{H}$  NMR:  $\delta$  8.82 (s, 1 H), 8.20-7.83 (m, 4 H), 7.5 (apparent t, 1 H), 7.25 (apparent t, 1 H), 4.36-4.15 (m, 4 H), 3.93 (s, 3 H), 3.48 (t,  $J = 6.9$  Hz, 4 H), 3.06-2.77 (m, 4 H), 2.86 (t,  $J = 6.9$  Hz, 4 H) The amine proton was not observed.  $^{13}\text{C}$  NMR:  $\delta$  172.3, 166.6, 155.2, 146.5, 144.6, 133.1, 131.6, 128.7, 124.6, 124.3, 116.1, 114.3, 63.7, 57.2, 53.5, 52.9, 46.3, 42.5, 35.2. No other carbons were observed. The HCl salt was precipitated from  $\text{CH}_2\text{Cl}_2$  by addition of 1 M HCl in ether to give  $\beta$ -Alanine, N-(2-carbomethoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride (Compound IV,) as a yellow solid (81 % pure by HPLC).

$\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride, (Compound V) was prepared in a similar manner. Thus using 9-methoxyacridine in place of methyl 9-methoxyacridine-2-carboxylate in Step D, the intermediate diol was obtained (7.1%) as a yellow oil (74% pure by HPLC).  $^1\text{H}$  NMR:  $\delta$  8.14 (d,  $J = 7.5$  Hz, 2 H), 7.93 (d,  $J = 8.6$  Hz, 2 H), 7.52 (apparent t, 2 H), 7.23 (apparent t, 2 H), 4.36-4.08 (m, 4 H), 3.76-3.5 (m, 4 H), 3.08-2.60 (m, 8 H) The amine and hydroxyl protons were not observed.

A solution of the intermediate diol (37.3 mg, 0.0793 mmol) in thionyl chloride (4.0 mL) was stirred at 23 °C for 7.5 h. The thionyl chloride was removed *in vacuo* to give a yellow oil. The material was dissolved in ethanol (~4 mL) and the solvent removed *in vacuo*. The material was then dissolved in  $\text{CH}_2\text{Cl}_2$  (4 mL) and solvent removed *in vacuo*; this step was repeated twice. The material was then triturated with hexane (3 x 4 mL) to give 40.0 mg (42 % pure by HPLC) of the product in the form of a yellow hygroscopic glassy solid. Some of the material was converted

to the free amine for analytical purposes by partitioning between saturated  $\text{NaHCO}_3$  and  $\text{CH}_2\text{Cl}_2$  followed by drying the combined organic layers over  $\text{Na}_2\text{SO}_4$  and removal of the solvent *in vacuo*.  
 $^1\text{H}$  NMR:  $\delta$  8.21-8.00 (m, 4 H), 7.66 (apparent t, 2 H), 7.38 (apparent t, 2 H), 4.26-4.12 (m, 2 H), 4.12-3.98 (m, 2 H), 3.43 (t,  $J = 6.9$  Hz, 4 H), 2.96-2.68 (m, 8 H) The amine proton was not  
5 observed.

Following the above procedure but replacing N-(*tert*-butoxycarbonyl)- $\beta$ -alanine with N-(*tert*-butoxycarbonyl)-4-aminobutyric acid led to the preparation of 4-aminobutyric acid N-[(2-carbomethoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride, Compound VI  
10 (78% pure by HPLC).  $^1\text{H}$  NMR:  $\delta$  8.89 (s, 1), 8.12 (apparent t, 2), 7.93-7.80 (m, 2), 7.59 (apparent q, 1), 7.36-7.20 (m, 1), 4.16 (t, 2,  $J = 5.7$  Hz), 4.07-3.92 (m, 2), 3.97 (s, 3), 3.46 (t, 4,  $J = 6.9$  Hz), 2.93-2.80 (m, 6), 2.60 (t, 2,  $J = 6.5$  Hz), 2.29-2.12 (m, 2) The amine proton was not observed.

### 15 Example 8

Substituting the triethanolamine in Example 7, Step A with 3-[N,N-Bis(2-*tert*-butyldimethylsilyloxyethyl)]aminopropanol, and then continuing from step C, led to the preparation of  $\beta$ -Alanine, N-(2-carbomethoxy-acridin-9-yl), 3-[bis(2-chloroethyl)amino]propyl  
20 ester dihydrochloride, Compound VIII, (63% pure by HPLC).

$^1\text{H}$  NMR :  $\delta$  8.91 (s, 1), 8.20-7.93 (m, 4), 7.18 (apparent t, 1), 7.39 (apparent t, 1), 4.30 (m, 4), 3.96 (s, 3), 3.48 (t, 4,  $J = 6.9$  Hz), 2.88-2.60 (m, 2), 2.83 (t, 4,  $J = 6.9$  Hz), 2.62 (t, 2,  $J = 6.7$  Hz), 1.85-1.68 (m, 2) The amine proton was not observed.

### 25 Example 9

The compounds synthesized in Example 7 can also be prepared by the following method:

Synthesis of  $\beta$  -Alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride (Compound V): Method II

30 Step A:  $\beta$  -Alanine, N-(acridin-9-yl), methyl ester hydrochloride

9-Chloroacridine (11.7 g, Organic Synthesis, Coll. Vol III, pg. 57),  $\beta$ -alanine methyl ester hydrochloride (9.9 g) and sodium methoxide (3.26 g) were combined and 60 mL of methanol was added. The mixture was stirred with a magnetic stirrer and refluxed for 5.5 h. Heat was removed and the suspension was filtered while warm ( $\leq 35^\circ\text{C}$ ). The solid salts were rinsed with about 10 mL of additional methanol and the combined dark green filtrate was concentrated to give 21 g of a moist greenish-yellow solid.

The solid was dissolved in 350 mL of boiling 2-propanol and allowed to crystallize at room temperature. The resulting crystals were rinsed with about 15 mL of 2-propanol and 15 mL of hexane, then air dried to give 15.5 g of bright yellow product,  $\beta$ -Alanine, N-(acridin-9-yl), methylester hydrochloride, (yield 78.5%).  $^1\text{H}$  NMR:  $\delta$  1.9 (br s, 2H); 3.24 (t,  $J=7.0$  Hz, 2H); 3.76 (s, 3H); 4.45 (br s, 2H); 7.23 (app. t,  $J=8$  Hz, 2H); 7.49 (app. t,  $J=8$  Hz, 2H); 8.11 (d,  $J=8.4$  Hz, 2H); 8.30 (d,  $J=8.4$  Hz, 2H); 9.68 (br s, 0.5 H). IR: 1574 (s), 1691 (s), 1726 (s), 2336 (m), 2361 (m), 3227 (m).

Step B:  $\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-hydroxyethyl)amino]ethyl ester dihydrochloride

The  $\beta$ -Alanine, N-(acridin-9-yl), methyl ester hydrochloride, from Step A, (5.00 g) was partitioned between toluene (750 mL), saturated aqueous  $\text{Na}_2\text{CO}_3$  (200 mL) and  $\text{H}_2\text{O}$  (50 mL). The aqueous layer was extracted again with toluene (3 x 250 mL) and the organic layers were combined and washed with saturated aqueous  $\text{Na}_2\text{CO}_3$  (50 mL). The volume of toluene was reduced to about 100 mL by rotary evaporation. Triethanolamine (30 mL) was then added to form a partially immiscible system. A solution of NaOMe (50 mg) in MeOH (2 mL) was then added. Solvents were quickly removed from the reaction mixture by rotary evaporation with agitation at room temperature. After the solvent was removed the reaction mixture was left under vacuum for another 1-1.5h to give a syrupy solution.

The crude mixture was partitioned between  $\text{CH}_2\text{Cl}_2$  (200 mL) and brine (200 mL) to remove excess triethanolamine. The brine layer was extracted with  $\text{CH}_2\text{Cl}_2$  (5 x 100 mL). The organic layers were combined and washed with brine (50 mL) then extracted with 0.5M HCl (2 x 100 mL). The aqueous acid layers were combined and washed with  $\text{CH}_2\text{Cl}_2$  (50 mL). The acid solution was made basic with powdered  $\text{K}_2\text{CO}_3(\text{s})$  in the presence of  $\text{CH}_2\text{Cl}_2$  (200 mL). The organic layer was separated and the aqueous layer was extracted again with  $\text{CH}_2\text{Cl}_2$  (5 x 100 mL). The combined organic layers were washed with brine (50 mL), dried with anhydrous

Na<sub>2</sub>SO<sub>4</sub>(s), and stripped to give crude diol free amine (5.02 g), a sticky yellow gum. This material was identical by NMR to that prepared in Example 1 by an alternate procedure.

A portion of the above crude (0.400g) was vigorously stirred with isopropanol (100 mL) and acidified with 1 M HCl in ether. The slurry was chilled and the first precipitate was discarded. After removing half the solvent the second set of crystals gave  $\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-hydroxyethyl)amino]ethyl ester dihydrochloride as a bright yellow crystalline solid (0.200g) >95 % pure by HPLC. <sup>1</sup>H NMR:  $\delta$  8.11 (apparent t, 4 H), 7.69 (apparent t, 2 H), 7.41 (apparent t, 2 H), 4.23 (t, J = 5.4 Hz, 2 H), 4.03 (t, J = 5.9 Hz, 2 H), 3.58 (t, J = 5.2 Hz, 4 H), 2.73 (t, J = 5.4 Hz, 2 H), 2.70 (t, J = 5.9 Hz, 2 H) 2.68 (t, J = 5.2 Hz, 4 H). The amine and hydroxyl protons were not observed. <sup>13</sup>C NMR:  $\delta$  173.3, 151.7, 149.4, 130.5, 129.5, 124.0, 123.4, 118.4, 63.5, 60.1, 57.3, 54.0, 46.6, 35.8.

Step C:  $\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride

SOCl<sub>2</sub> (0.5 mL) was added to a stirred suspension of  $\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-hydroxyethyl)amino]ethyl ester dihydrochloride from Step B (113 mg, 0.24 mmol) in CH<sub>3</sub>CN (0.5 mL). The resultant yellow solution was stirred at 23 °C for 16 h followed by removal of the volatiles *in vacuo*. The remaining orange oil was dissolved in EtOH (~2 mL) and the EtOH was removed *in vacuo* to give a yellow solid. The material was then triturated with hexane (2 x 3 mL). Removal of residual solvents *in vacuo* gave 123 mg of the desired material,  $\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride, (93 % pure by HPLC) as a yellow solid. <sup>1</sup>H NMR:  $\delta$  8.09 (apparent t, J = 8.8 Hz, 4 H), 7.66 (apparent t, J = 7.6 Hz, 2 H), 7.38 (apparent t, J = 7.7 Hz, 2 H), 4.14 (t, J = 5.9 Hz, 2 H), 4.00 (t, J = 5.8 Hz, 2 H), 3.43 (t, J = 6.9 Hz, 4 H), 2.87 (t, J = 6.9 Hz, 4 H), 2.77 (t, J = 5.9 Hz, 2 H), 2.69 (t, J = 5.8 Hz, 2 H). The amine proton was not observed. <sup>13</sup>C NMR :  $\delta$  173.0, 151.5, 149.4, 130.5, 129.6, 124.1, 123.4, 118.6, 63.5, 57.3, 53.5, 46.7, 42.5, 35.7. IR (KBr pellet of HCl salt): 3423, 3236, 2939, 2879, 1736, 1634, 1586, 1572, 1540, 1473, 1272, 1173 cm<sup>-1</sup>.

### Example 10

$\beta$ -alanine, N-(4-methoxy-acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride, Compound IX.

$\beta$ -alanine, N-(4-methoxy-acridin-9-yl), methyl ester was prepared by mixing 1.4 g (5.84 mmol) of 4,9-dimethoxyacridine, 0.89 g (6.42 mmol) of  $\beta$ -alanine methyl ester hydrochloride and 20 mL of methanol and then heating to reflux for 12h under N<sub>2</sub>. The reaction was then concentrated *in vacuo*, dissolved in CHCl<sub>3</sub>-isopropanol (50 mL, 4:1 v/v), and washed with 50% NH<sub>4</sub>OH (2 x 25 mL) and brine (1 x 25 mL). The organic layer was dried with Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to yield 1.24 g (68%) of the methyl ester (>74% purity by HPLC) as a yellow oil; R<sub>f</sub> (SiO<sub>2</sub>, ethyl acetate) = 0.25; IR (thin film): 3363, 2947, 1730, 1611, 1573, 1518, 1484, 1463, 1423, 1420, 1246, 1170, 1081 cm<sup>-1</sup>; <sup>1</sup>H NMR:  $\delta$  2.70 (t, 2H, *J*=5.7 Hz), 3.74 (s, 3H), 4.00 (t, 2H, *J*=6.3 Hz), 4.11 (s, 3H), 6.98 (d, 1H, *J*=7.4 Hz), 7.36 (m, 2H), 7.65 (m, 2H), 8.12 (d, 2H, *J*=8.5 Hz); <sup>13</sup>C NMR:  $\delta$  35.7, 46.9, 52.3, 56.5, 107.2, 115.3, 119.8, 123.5, 124.1, 130.0, 151.4, 173.6.

This was converted to the diol under conditions described in Example 3, Step B to afford 647 mg of a yellow oil. HPLC analysis of the crude mixture indicated a yield of 85% ( $\lambda$  = 278 nm); R<sub>f</sub> (SiO<sub>2</sub>, 20% methanol-ethyl acetate) = 0.17; IR (thin film): 3337, 2947, 2828, 1726, 1616, 1569, 1522, 1484, 1463, 1420, 1348, 1250, 1174, 1127, 1081, 1043 cm<sup>-1</sup>; <sup>1</sup>H NMR:  $\delta$  2.7 (m, 8H), 3.55 (m, 4H), 3.97-4.08 (m, 2H), 4.08 (s, 3H), 4.19 (t, 2H, *J*=5.5 Hz), 6.96 (d, 1H, *J*=7.4 Hz), 7.29 (m, 2H), 7.61 (m, 2H), 8.10 (m, 2H); <sup>13</sup>C NMR:  $\delta$  36.0, 46.9, 53.7, 56.4, 57.1, 60.1, 63.3, 107.4, 115.7, 119.1, 119.6, 123.2, 123.5, 123.9, 128.5, 130.0, 140.8, 147.4, 151.6, 151.7, 154.3, 173.3.

This was converted to  $\beta$ -alanine, N-(4-methoxy-acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride with thionyl chloride as described in Example 3, Step C. Flash filtration (SiO<sub>2</sub>) of the crude product using ethyl acetate followed by 10% methanol-ethyl acetate gave 58 mg of a yellow oil after apparent on-column degradation of some product; R<sub>f</sub> (SiO<sub>2</sub>, ethyl acetate) = 0.26; IR (thin film): 3405, 2955, 2828, 1726, 1616, 1577, 1518, 1463, 1416, 1348, 1246, 1174, 1123, 1081, 1013 cm<sup>-1</sup>; <sup>1</sup>H NMR:  $\delta$  2.69-2.99 (m, 8H), 3.45 (t, 4H, *J*=6.7 Hz), 4.03 (m, 2H), 4.09 (s, 3H), 4.16 (t, 2H, *J*=5.9 Hz), 6.97 (d, 1H, *J*=7.7 Hz), 7.32 (m, 2H), 7.65 (m, 2H), 8.12 (d, 2H, *J*=8.7 Hz).

The dihydrochloride salt could be isolated in crude form by concentrating the reaction *in vacuo* with azeotropic removal of excess thionyl chloride (2 x 5 mL toluene). HPLC analysis indicated complete consumption of the starting material and 4-methoxy acridone (R<sub>T</sub>=22.3 min) to be the major impurity. <sup>1</sup>H NMR (CD<sub>3</sub>OD):  $\delta$  3.18 (t, 2H, *J*=6.4 Hz), 3.71 (m, 6H), 4.04 (m,

4H), 4.18 (s, 3H), 4.51 (m, 2H), 7.17 (m, 2H), 7.56 (m, 2H), 7.91-8.15 (m, 2H), 8.55 (d, 1H,  $J=8.8$  Hz).

Similarly prepared from 3-chloro-9-methoxy-4-methylacridine was  $\beta$ -alanine, N-(3-chloro-4-methylacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride, Compound X.  $^1\text{H}$  NMR of the free base:  $\delta$  7.96-8.17 (m, 3H), 7.29-7.52 (m, 3H), 4.19 (t,  $J = 5.8$  Hz, 2H), 4.00 (s, 3H), 3.89 (t,  $J = 5.1$  Hz, 2H), 3.47 (t,  $J = 6.8$  Hz, 4H), 2.91 (t,  $J = 6.8$  Hz, 4H), 2.83 (t,  $J = 5.8$  Hz, 2H), 2.67 (t,  $J = 5.5$  Hz, 2H).

Similarly prepared from 6-chloro-2,9-dimethoxyacridine was  $\beta$ -alanine, N-(6-chloro-2-methoxyacridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester dihydrochloride, Compound XIII.

$^1\text{H}$  NMR of the free base:  $\delta$  7.96-8.17 (m, 3H), 7.29-7.52 (m, 3H), 4.19 (t,  $J = 5.8$  Hz, 2H), 4.00 (s, 3H), 3.89 (t,  $J = 5.1$  Hz, 2H), 3.47 (t,  $J = 6.8$  Hz, 4H), 2.91 (t,  $J = 6.8$  Hz, 4H), 2.83 (t,  $J = 5.8$  Hz, 2H), 2.67 (t,  $J = 5.5$  Hz, 2H).

### Example 11

$\beta$ -Alanine, [N,N-bis(2-chloroethyl)], 3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl ester dihydrochloride, Compound XI.

Step A  $\beta$ -Alanine, [N, N-bis(2-triisopropylsilyloxy)ethyl]ethyl ester

A slurry of  $\beta$ -alanine ethyl ester hydrochloride (1.99 g, 12.9 mmol),  $\text{K}_2\text{CO}_3$  (6.0g, 43.4 mmol) and iodoethyl triisopropylsilyl ether (9.47 g, 28.9 mmol) in acetonitrile (175 mL) were refluxed for 5-7 days. After vacuum evaporation of the solvent, the solid was triturated with  $\text{CH}_2\text{Cl}_2$ . The organic layer was washed with dilute  $\text{Na}_2\text{CO}_3(\text{aq.})$ , then with brine and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The crude product was purified by silica gel chromatography (1:4 EtOAc / hexane) to provide 5.60 g of the oil,  $\beta$ -Alanine, [N, N-bis(2-triisopropylsilyloxy)ethyl]ethyl ester, (83.1%).  $^1\text{H}$  NMR:  $\delta$  4.12 (q,  $J = 7.1$  Hz, 2H), 3.73 (t,  $J = 6.8$  Hz, 4H), 2.92 (t,  $J = 7.3$  Hz, 2H), 2.70 (t,  $J = 6.6$  Hz, 4H), 2.46 (t,  $J = 7.4$  Hz, 2H), 1.4 - 0.9 (m, 45H, includes triplet at 1.25 (3H) and singlets at 1.06 and 1.05).

Step B  $\beta$ -Alanine, [N, N-bis(2-triisopropylsilyloxy)ethyl]-



The  $\beta$ -Alanine, [N, N-bis(2-triisopropylsilyloxy)-ethyl]ethyl ester from Step A above (5.60 g, 10.8 mmol) and lithium hydroxide (0.59 g, 14.1 mmol) were stirred in ethanol and refluxed for 3h. The solvent was removed and the crude product was partitioned between  $\text{CH}_2\text{Cl}_2$  and dilute  $\text{NaHCO}_3(\text{aq.})$ . The organic layer was washed with brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and  
5 stripped to give  $\beta$ -Alanine, [N, N-bis(2-triisopropylsilyloxy)ethyl]- as a pale yellow oil (5.03 g, 95.1% yield).  $^1\text{H}$  NMR:  $\delta$  3.90 (t,  $J$  = 5.5 Hz, 4H), 3.04 (t,  $J$  = 6.2 Hz, 2H), 2.92 (t,  $J$  = 5.5 Hz, 4H), 2.50 (t,  $J$  = 6.1 Hz, 2H), 1.06 (s, 42H).

Step C  $\beta$ -Alanine, [N,N-bis(2-hydroxyethyl)], 3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl  
10 ester

The  $\beta$ -Alanine, [N, N-bis(2-triisopropylsilyloxy)ethyl]- from Step B above (51.0 mg, 0.104 mmol) was stirred under  $\text{N}_2$  in  $\text{CH}_2\text{Cl}_2$  (1 mL). While chilling on an ice bath,  $\text{SOCl}_2$  (0.5 mL) was added dropwise and the reaction was stirred for 2.25 h. After stripping the reaction  
15 mixture to remove excess  $\text{SOCl}_2$ , dry  $\text{CH}_2\text{Cl}_2$  (0.5 mL) was added and the solution was chilled in an ice bath while under  $\text{N}_2$ . A chilled slurry of 9-(3-hydroxy)propylamino-6-chloro-2-methoxy-acridine (29.0 mg, 91.5 mmol) in  $\text{CH}_2\text{Cl}_2$  (1 mL) was added. After 0.5 h the mixture was partitioned between  $\text{CH}_2\text{Cl}_2$  and aqueous  $\text{NaHCO}_3$ . The organic layer was washed with brine, dried with anhydrous  $\text{Na}_2\text{SO}_4$ , and stripped. The gum obtained was triturated with hexane and  
20 the hexane extract was stripped to obtain a very crude mixture (53.5 mg) of triisopropylsilyl protected starting material and product.

To remove the triisopropylsilyl groups, a portion of the crude protected diol (33.1 mg) was stirred in ice cold THF (1 mL). After the addition of HF/pyridine (0.5 mL) the mixture was stirred at ambient temperature under a  $\text{N}_2$  filled balloon for 2.5 h. The reaction mix was partitioned  
25 between  $\text{CH}_2\text{Cl}_2$  and  $\text{NaHCO}_3(\text{aq.})$  and the organic layer was washed several times with dilute  $\text{NaHCO}_3(\text{aq.})$  to remove excess HF/pyridine. After preliminary drying with brine, then with anhydrous  $\text{Na}_2\text{SO}_4$ , the solvent was stripped off to give crude diol (13.1 mg).

This was combined with additional crude diol (5.0 mg) and purified by C-18 preparative TLC with 95  $\text{CH}_2\text{Cl}_2$  / 5 iPA / 1 TFA as eluent to obtain the diol TFA salt. After partitioning the  
30 salt between  $\text{CH}_2\text{Cl}_2$  and  $\text{NaHCO}_3(\text{aq.})$ , the organic layer was dried with brine, then with anhydrous  $\text{Na}_2\text{SO}_4$ , and stripped to give the free base of the diol,  $\beta$ -Alanine, [N,N-bis(2-

hydroxyethyl)], 3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl ester, (5.0 mg).  $^1\text{H}$  NMR:  $\delta$  7.92-8.25 (m, 3H), 7.23-7.47 (m, 3H), 4.30 (t,  $J = 5.7$  Hz, 2H), 3.98 (s, 3H), 3.81 (t,  $J = 6.2$  Hz, 2H), 3.64 (t,  $J = 4.9$  Hz, 4H), 2.86 (t,  $J = 6.1$  Hz, 2H), 2.67 (t,  $J = 4.9$  Hz, 4H), 2.51 (t,  $J = 5.9$  Hz, 2H), 2.04 (apparent quintet, 2H).

5

Step D  $\beta$ -Alanine, [N,N-bis(2-chloroethyl)], 3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl ester dihydrochloride, Compound XI.

10

The  $\beta$ -Alanine, [N,N-bis(2-hydroxyethyl)], 3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl ester from above (4.0 mg, 0.0073 mmol) was dissolved in  $\text{CH}_2\text{Cl}_2$  (1 mL) and chilled in an ice/water bath. Ice cold  $\text{SOCl}_2$  (0.1 mL) was added and the reaction was allowed to stir for 4 h at room temperature. The reaction mixture was stripped to remove solvent, triturated with hexane, and partitioned between  $\text{CH}_2\text{Cl}_2$  and  $\text{NaHCO}_3(\text{aq.})$ . After the organic layer was dried with brine, then with anhydrous  $\text{Na}_2\text{SO}_4$  and stripped, the dichloro- compound was obtained as a yellow gum.  $^1\text{H}$  NMR:  $\delta$  7.8-8.2 (m, 3H), 7.2-7.5 (m, 3H), 4.35 (t,  $J = 5.9$  Hz, 2H), 3.85-4.10 (3.99, s, OMe and 3.9-4.0, m,  $\text{NHCH}_2$ , total 5H), 3.48 (t,  $J = 6.9$  Hz, 4H), 2.9-3.0 (m, 6H), 2.49 (t,  $J = 6.6$  Hz, 2H), 2.1-2.3 (m, 2H).

15

The free amine was stirred in chilled  $\text{CH}_2\text{Cl}_2$ , acidified with 1M HCl in ether and stripped with a few drops of methanol to obtain the desired compound,  $\beta$ -Alanine, [N,N-bis(2-chloroethyl)], 3-[(6-chloro-2-methoxyacridin-9-yl)amino]propyl ester dihydrochloride (2.5 mg), (3.5 mg, 81 %), as a yellow solid.

20

In the same manner as given in the foregoing Step C, but using 6-chloro-9-(2-hydroxy)ethylamino-2-methoxy-acridine instead of 6-chloro-9-(3-hydroxy)propylamino-2-methoxy-acridine, was prepared the analogous diol.  $^1\text{H}$  NMR :  $\delta$  7.96-8.13 (m, 3H), 7.20-7.47 (m, 3H), 4.76 (t,  $J = 4.9$  Hz, 2H), 3.99 (s, 3H), 3.92-4.14 (m, 2H), 3.60 (t,  $J = 5.1$  Hz, 4H), 2.78 (t,  $J = 6.1$  Hz, 2H), 2.63 (t,  $J = 5.1$  Hz, 4H), 2.45 (t,  $J = 6.0$  Hz, 2H). By analogy to Step D this was converted to  $\beta$ -Alanine, [N,N-bis(2-chloroethyl)], 2-[(6-chloro-2-methoxyacridin-9-yl)amino]ethyl ester dihydrochloride, Compound XII.  $^1\text{H}$  NMR:  $\delta$  7.94-8.20 (m), 7.20-7.50 (m), 4.42 ( $\text{CH}_2\text{OC}=\text{O}$ ), 3.90-4.10 ( $\text{OCH}_3$ ,  $\text{NHCH}_2$ ), 3.46 ( $\text{CH}_2\text{Cl}$ ), 2.82 ( $\text{N}(\text{CH}_2)_3$ ), 2.39-2.56 ( $\text{CH}_2\text{C}=\text{O}$ ).

25

30

### Example 12

[N,N-Bis(2-chloroethyl)]-2-aminoethyl 4,5',8-trimethyl-4'-psoralenacetate hydrochloride, Compound XIV

Step A: [N,N-Bis(2-hydroxyethyl)]-2-aminoethyl 4,5',8-trimethyl-4'-psoralenacetate

5           A slurry of methyl 4,5',8-trimethyl-4'-psoralenacetate (250 mg, 0.832 mmol), triethanolamine (12 mL) and 1M HCl in ether (2 mL) were stirred at 100°C for 2 h. The resulting clear brown solution was allowed to cool to room temperature and partitioned between CH<sub>2</sub>Cl<sub>2</sub> and saturated NaHCO<sub>3</sub>(aq.). The organic layer was rinsed several times with saturated NaHCO<sub>3</sub>(aq.). After drying with anhydrous Na<sub>2</sub>SO<sub>4</sub>, solvent was removed  
10 *in vacuo* and the residue was partitioned between CH<sub>2</sub>Cl<sub>2</sub> and 1M aq. HCl. The aqueous layer was rinsed several times with CH<sub>2</sub>Cl<sub>2</sub> and then made basic with K<sub>2</sub>CO<sub>3</sub>(s) in the presence of the organic solvent. The organic layer containing the neutral product was rinsed with water several times, then dried and concentrated. A repetition of the acid-base extraction procedure gave the desired product as a beige solid (84.3 mg, 24.3%): <sup>1</sup>H  
15 NMR: δ 7.53 (s, 1H), 6.24 (s, 1H), 4.23 (t, J = 5.4 Hz, 2H), 3.69 (s, 2H), 3.56 (t, J = 5.3 Hz, 4H), 2.82 (t, J = 5.4 Hz, 2H), 2.69 (t, J = 5.3 Hz, 4H), 2.57 (s, 3H), 2.51 (d, J = 1.1 Hz, 3H), 2.47 (s, 3H).

Step B: [N,N-Bis(2-chloroethyl)]-2-aminoethyl 4,5',8-trimethyl-4'-psoralenacetate  
20 hydrochloride

Thionyl chloride (0.2 mL) was added to an ice cold mixture of the above diol (9.8 mg, 0.023 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (1 mL) and stirred at room temperature overnight under nitrogen. The resulting slurry was concentrated then triturated with hexane to give the desired product (6.2 mg, 53.9%) as an off-white solid: <sup>1</sup>H NMR (CD<sub>3</sub>OD): δ 7.71 (s, 1H),  
25 6.28 (s, 1H), 4.56 (t, J = 4.8 Hz, 2H), 3.95 (t, J = 6.1 Hz, 4H), 3.89 (s, 2H), 3.60-3.83 (m, 6H), 2.54 (s, 3H), 2.53 (s, 3H), 2.50 (s, 3H).

### Example 13

#### **Exemplary BMT light or minitransplant protocol**

The patient presenting CLL is given a nonablative preparative regimen prior to  
30 transplantation with allogeneic peripheral blood stem cells. Transplantation is followed by support with DLI as necessary to achieve a maximum graft-versus-malignancy effect. The mildly toxic preparative regimen is fludarabine (FAMP)-based and is modified according to malignancy type. For advanced and previously treated CLL, the patient is given FAMP

at 300 mg/m<sup>2</sup>/dx3 with cyclophosphamide at 30 mg/m<sup>2</sup>/dx3. Richter and Large Cell Lymphoma (LCL) are treated with a regimen consisting of FAMP 30 mg/m<sup>2</sup>/dx2, cisplatin 25 mg/m<sup>2</sup>/d/C1x4, and Ara-c 0.5 gm mg/m<sup>2</sup>/dx2. With the exception of treatment for LCL, no GVHD prophylaxis is given. The regimen is intended to generate chimerisms and allow engraftment while decreasing the toxicity of conventional induction therapy. DLI can be given 1 month post-transplant to boost engraftment. Successful engraftment allows for subsequent DLI administered as necessary to enhance a graft vs. leukemia effect and expedite immune recovery.

#### **Example 14**

##### ***In vitro* studies with human peripheral blood lymphocytes**

To characterize a particular type of treated cell population, several features of human peripheral blood lymphocytes (PBLs), subjected to PCT and cultured *in vitro*, were examined. These included viability, T-cell proliferation, cytokine synthesis and secretion, surface antigen expression, *in vitro* cytotoxicity and Fas ligand expression.

##### **Viability**

PBLs subjected to 10 nM S-59 at 3 J/cm<sup>2</sup> UVA were assayed for viability by trypan blue exclusion. Survival was 75% after 2 days, 50% after three days, and 25% after 4 days. In related experiments, a dose-dependent survival of treated lymphocytes was observed.

##### **Proliferation**

Lymphocyte proliferation was measured by <sup>3</sup>H-thymidine incorporation into the DNA of allostimulated T-cells. Peripheral blood mononuclear cells (PBMCs) were isolated from three donors, pooled, γ-irradiated (2500 cGy) to prevent autostimulation, and used as stimulator cells in a MLR. PBMCs isolated from a fourth donor were used as responder cells. Responder cells were treated with 0.05, 0.5 or 5 nM 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen ("S-59") at 3 J/cm<sup>2</sup> UVA and co-cultured with stimulator cells. Control, untreated responder cells were also co-cultured with the stimulator cells. After 6 days of culture, <sup>3</sup>H-thymidine was added to the cultures, and, one day later, cells were harvested and cell samples were analyzed by scintillation counting to measure uptake of <sup>3</sup>H thymidine. Figure 8 shows that proliferation of treated leukocytes, as measured by

<sup>3</sup>H-thymidine uptake in a MLR, was eliminated in a dose-dependent manner between 0.1 nM and 10 nM S-59, with complete inhibition of proliferation at 10 nM S-59.

Proliferative ability of treated leukocytes was also analyzed by an alternative method, in which treated cells were activated by surface-bound anti-CD3 antibody. In this assay, PBMCs were either untreated, treated with 0.0001, 0.001 or 0.01  $\mu$ M S-59 at a UVA dose of 3 J/cm<sup>2</sup>, or irradiated in the absence of S-59. Cells were then incubated at 37°C in 5% CO<sub>2</sub> in plates to which anti-CD3 antibody had been attached, to induce polyclonal activation of T-cells, and <sup>3</sup>H-thymidine incorporation was measured after 1, 2 and 3 days of culture. The results, (Figure 9) show a dose-dependent reduction in proliferative capacity under all treatment conditions, and complete inhibition of proliferation at 10 nM S-59.

### Cytokine production

Human lymphocytes were cultured in a MLR as described in the previous section on analysis of proliferation. Responder cells were treated with different concentrations of S-59 (0.05, 0.5 or 5 nM) at 3.0 J/cm<sup>2</sup> UVA. Samples of the MLR cell culture medium were taken after 2, 4, 6, and 7 days of culture. These samples were tested for IL-2 and IFN- $\gamma$  levels by sandwich enzyme-linked immunosorbent assay and the results were quantitated by spectrophotometry. Standard solutions were used for the construction of standard curves relating concentration (pg/ml) and absorbance, from which cytokine concentrations in the test samples were determined.

Analysis of IL-2 production is shown in Figure 10A. Secretion of IL-2 by untreated lymphocytes and by lymphocytes treated with lower concentrations of S-59 (*i.e.*, 0.05 and 0.5 nM) peaked at 2 days in culture, and then declined due to consumption of IL-2 by proliferating lymphocytes in the population. By contrast, in lymphocytes treated with 5 nM S-59, IL-2 was not consumed by the non-proliferating leukocytes, and remained present in the medium, at fairly high levels, at days 4, 6 and 7.

The production of IFN- $\gamma$  by stimulated PBMCs was unaffected or somewhat increased by treatment with S-59 + UVA. *See* Figure 10B. The combined results of the IL-2 and IFN- $\gamma$  analyses show that, under conditions in which proliferation is inhibited, treated cells are nevertheless capable of synthesizing and secreting cytokines associated with T-cell activation.

### Surface antigen expression

Analysis of surface marker expression on treated lymphocytes was analyzed. Lymphocytes were obtained by Ficoll gradient centrifugation, and were activated through *in vitro* stimulation by surface-bound anti-CD3 antibody. Surface antigen expression by activated, treated lymphocytes was measured by fluorescence-activated cell sorting (FACS) analysis, using fluorescent antibodies to CD69, CD25 (the IL-2 receptor), or CD40L. Expression was measured as a function of time after activation and compared to expression by untreated cells.

CD69 is an early marker of lymphocyte activation. Figure 11A shows that cells treated with either 1 nM or 10 nM S-59 + 3 J/cm<sup>2</sup> UVA have surface CD69 levels similar to, or slightly higher than, those of untreated cells, for up to 48 hours post-treatment. Thus, although treated cells are unable to proliferate, signalling pathways related to activation remain functional.

CD 25 is the IL-2 receptor, and its expression is detected somewhat later (after activation) than that of CD69. Figure 11B shows that, in cells treated with either 1 nM or 10 nM S-59 + 3 J/cm<sup>2</sup> UVA, surface CD25 levels are essentially unaffected, compared to untreated cells.

The CD40 ligand (CD40L) on T-cells recognizes the CD40 molecule expressed on the surface of B-cells as part of the T-cell activation process. Inhibition of CD40L expression on T-cells can induce cell unresponsiveness or anergy. Therefore, unperturbed expression of CD40L on T-cells after treatment is an indication of normal T-cell activation. The effect of PCT on expression of CD40L was examined following activation of treated cells with anti-CD3 antibody (Figure 12). The results of this analysis indicate that, for treatment conditions under which subsequent proliferative ability is abolished, CD40L expression by treated cells closely parallels that observed in untreated cells.

In conclusion, under conditions in which proliferation is severely reduced or completely inhibited (see Figures 8 and 9, this Example), expression of the early T-cell activation marker CD69, and the IL-2 receptor CD25 remain essentially unaffected; and CD40L levels parallel those of untreated cells. It should be noted that CD 69 is transiently expressed on lymphocytes upon antigenic or mitogenic stimulation and continuous expression of CD69 requires new transcription. Thus, observation of high CD69 levels in treated, nonproliferating cells further supports the notion that treatment does not irreversibly inhibit gene expression.

### **Cytotoxic T-cell function**

The effect of S-59 + UVA treatment of the lytic function of T-cells was evaluated by generating cytotoxic T-cells against gamma irradiation-inactivated allogeneic  
5 “stimulator” cells in a MLR. These cytotoxic T-cells were then tested for their ability to lyse  $^{51}\text{Cr}$ -labeled target cells that were obtained from the same donor used to provide the inactivated stimulator cells.

#### Generation of cytotoxic T-cells

Peripheral blood (100 ml) was removed from two donors, one designated the  
10 “stimulator” and the other, the “responder.” PBMCs were isolated from both populations by Ficoll gradient centrifugation. Responder PBMCs were depleted of monocytes by placing them in a tissue culture flask, at a cell density of  $5 \times 10^6$  cells/ml, for one hour. Stimulator PBMCs were  $\gamma$ -irradiated (2500cGy) to block cell division. Both the monocyte-depleted responders and the irradiated stimulators were separately resuspended  
15 in RPMI complete medium to a concentration of  $1 \times 10^6$  cells/ml. Equal volumes of responder and stimulator cell suspensions were then combined for MLR, and incubated at  $37^\circ\text{C}$  for 7 days in a 5%  $\text{CO}_2$  incubator.

#### Preparation of target cells

Peripheral blood (50 ml) was drawn from the same donor that provided the  
20 stimulator cells for MLR. PBMCs were isolated as above and resuspended at a concentration of  $1 \times 10^6$  cells/ml in RPMI medium, then stimulated with 2  $\mu\text{g}/\text{ml}$  PHA-M (phytohemagglutinin-M) and 10 Units/ml of recombinant IL-2 for 7 days at  $37^\circ\text{C}$ .

#### Labeling of target cells with $^{51}\text{Cr}$

Target cells were incubated in the presence of 200  $\mu\text{Ci}$   $\text{Na}_2^{51}\text{CrO}_4$  at  $37^\circ\text{C}$  for two  
25 hours, then washed three times to remove unincorporated label.

#### Photochemical treatment (PCT) of Responder cells

Responder cells (monocyte-depleted, as described above) were divided into four equal portions, each of which was resuspended in 30 ml of PBS containing 1% bovine serum albumin. S-59 was added to three of the portions to a final concentration of 0.1,  
30 0.01 or 0.001  $\mu\text{M}$ ; the remaining portion served as an untreated control. Samples containing S-59 were transferred into 30 ml 2410 blood bags and each was illuminated with 3  $\text{J}/\text{cm}^2$  UVA on a Baxter/Fenwall UVA illumination device.

### CTL Assay

Cytotoxic T-lymphocyte (CTL) activity of the treated responder cell population was assayed as follows. The treated cells (or control untreated cells) were resuspended to a final concentration of  $5 \times 10^6$  cells/ml in a final volume of 1 ml, and serial two-fold dilutions were made to obtain final concentrations of  $2.5 \times 10^6$ ,  $1.25 \times 10^6$  and  $0.625 \times 10^6$  cells/ml. A 100  $\mu$ l sample of the resuspended treated responder cells and of each treated responder cell dilution ("effector cells") was added to each of three wells of a round-bottom 96-well microtiter plate. A 100  $\mu$ l sample of labeled target cells (prepared as described above), containing  $5 \times 10^3$  cells, was then added to each well of effector cells, to give effector cell:target cell ratios of 100:1, 50:1, 25:1 and 12.5:1, and the mixtures were incubated at 37°C for 4 hours. After the incubation, the plate was centrifuged for 5 min at 250 x g, and 100  $\mu$ l of supernatant was removed from each well. The amount of  $^{51}\text{Cr}$  in the supernatants was quantitated on a gamma counter. Average  $^{51}\text{Cr}$  cpm, and standard deviation, for each triplicate set of wells was calculated.

### Controls

Unlabeled target cells were diluted and plated with labeled target cells, at the same ratios as the effector cells, to measure spontaneous release of  $^{51}\text{Cr}$ . Labeled target cells were also incubated with RPMI and 1% Triton X-100 (3 wells each) to measure maximal release of  $^{51}\text{Cr}$ . Average  $^{51}\text{Cr}$  cpm, and standard deviation, for each triplicate set of wells was calculated.

### Results

Figure 13 shows data for release of  $^{51}\text{Cr}$  at different effector:target (or unlabeled target: labeled target) ratios for treated and untreated leukocytes. These results indicate that cytolytic function is retained by leukocytes that have been treated with doses of S-59 and UVA that block proliferation. Thus, treated leukocytes that are unable to proliferate not only possess the same phenotype as untreated cells (as shown by surface marker and cytokine expression) but they also possess similar functional properties.

In summary, analysis of proliferation, surface marker expression, cytokine synthesis and CTL activity of treated leukocyte populations has revealed the following properties of the treated leukocytes:

1. Upon treatment, a dose-dependent inhibition of proliferative ability is observed.
2. Cytokine synthesis, cell surface antigen expression and in vitro cytotoxicity are retained by treated cells.



Accordingly, populations of leukocytes treated as described herein, and equivalent cell populations, can facilitate engraftment of donor cells, for example, hematopoietic cells and hematopoietic stem cells, in nonmyeloablated hosts. One mechanism by which this may be achieved is through induction of donor-specific tolerance by the treated leukocyte population.

### **Example 15**

#### **Provision of GVL effect in the absence of GVHD by treated leukocytes in a murine model system**

Experiments were carried out to study the effects of S-59 + UVA treatment on the GVL activity of donor lymphocytes in a MHC-mismatched B6/AKR murine model system. Donor splenocytes were passed through a fine mesh screen to obtain single-cell suspensions of unseparated splenocytes and were then treated with 0.01  $\mu\text{M}$  S-59 and varying doses of UVA (0.5 min, 1 min, 2 min and 8 min).  $1 \times 10^7$  treated donor splenocytes, along with  $5 \times 10^6$  T-cell-depleted bone marrow cells (C57BL/6, H-2b, Thy 1.2<sup>+</sup>) were injected into AKR, H-2k, Thy 1.1<sup>+</sup> hosts that had been subjected to 1100 R of total body irradiation. Each group of mice (6 animals per group) was challenged with 250 AKR-M2 leukemia cells 3 days after transplant. Transplanted animals were observed for clinical symptoms of GVHD, leukemia relapse and death. In addition, body weight of the transplanted animals was recorded every 3-4 days after transplant.

The results, summarized in Table 3 and Figures 14 and 15, demonstrate that appropriate ranges of UVA + S-59 dose, effective in preserving GVL activity while decreasing GVHD in MHC-mismatched transplants, can be obtained. For example, addition of untreated or mildly treated (0.5 min UVA + 0.01  $\mu\text{M}$  S-59; exposure dose = 1.8 J-nM/cm<sup>2</sup>) lymphocytes to T-cell-depleted bone marrow resulted in mild to severe GVHD in transplanted recipients (as evidenced by the decreased body weight in these groups of animals; Figure 14). In contrast, lymphocytes treated with 1 min of UVA + 0.01  $\mu\text{M}$  S-59 (exposure dose = 3.7 J-nM/cm<sup>2</sup>) were effective in preserving their GVL activity (3 of 3 survivors at 70 days post-leukemia challenge) with no signs of GVHD. See Table 3 and Figure 15. Body weight of animals treated with 1 min of UVA + 0.01  $\mu\text{M}$  S-59 (Figure 14) also indicated lack of GVHD. The GVL activity of donor lymphocytes was abolished if cells were treated, at the same S-59 concentration, with higher UVA doses (2 and 8 min

UVA, equivalent to exposure doses of 7.5 and 30 J-nM/cm<sup>2</sup>, respectively).. Under these conditions, 3/3 mice died within 20 days of transplant. *See Figure 15*

**Table 3**  
**GVL Activity in B6/AKR chimeras administered PCT leukocytes**

Group	Treatment		Leukemia challenge:		Health Status
			None	250 cells	
1	0.01 $\mu$ M S-59 8 min UVA	# alive: MST: Range:	3/3 >70 X, X, X	1/3 18 18, 18, 19	Survivors healthy
2	0.01 $\mu$ M S-59 2 min UVA	# alive: MST: Range:	3/3 >70 X, X, X	1/3 18 18, 18, 21	Survivors healthy
3	0.01 $\mu$ M S-59 1 min UVA	# alive: MST: Range:	3/3 >70 X, X, X	3/3 >70 X, X, X	Healthy: cannot distinguish leukemia-challenged from unchallenged mice
4	0.01 $\mu$ M S-59 0.5 min UVA	# alive: MST: Range:	2/3 >70 58, X, X	3/3 >70 X, X, X	Severe GVHD in both leukemia-challenged and unchallenged mice
5	0.01 $\mu$ M S-59 No UVA	# alive: MST: Range:	3/3 >70 X, X, X	3/3 >70 X, X, X	Mild GVHD
6	BMT only	# alive: MST: Range:	4/4 >70 X, X, X, X	0/5 18 17, 18, 18, 18, 19	Healthy

**Notes:** - All groups were irradiated on day -1 and received MHC-mismatched bone marrow transplants, and treated splenocytes (if applicable), on day 0.  
- Leukemia challenge, if done, was on day 3.  
- MST = median survival time, in days  
- Range = day (after transplant) of death (X = alive)

From these results, it can be concluded that addition of leukocytes treated with appropriate doses of S-59 + UVA to T-cell-depleted bone marrow can prevent GVHD in a major MHC-mismatched murine model system. Furthermore, addition of appropriately treated leukocytes facilitates donor engraftment and leads to stable chimerism in a MHC-mismatched transplanted host. Finally, the GVL activity of treated leukocytes is preserved. Thus, treatment of leukocytes with S-59 + UVA is a novel method for modulating immunological activities of donor lymphocytes to aid allogeneic transplantation.

### Example 16

#### **The effect of S-303 on human lymphocyte proliferation and T-cell function**

Measurements were conducted on treated, allostimulated T-cells in a MLR assay. Peripheral blood mononuclear cells (PBMCs) were isolated from a donor, gamma-irradiated at a dose of 2,500 cGy to prevent autostimulation, and used as stimulator cells. PBMCs isolated from a different donor were used as responder cells. Responder cells at a concentration of  $2 \times 10^6$  cells/ml were treated with 0.1, 0.2, 0.3, 0.4, or 0.5  $\mu$ M  $\beta$ -Alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester (S-303) at room temperature for approximately 15 min and washed twice with PBS containing 1% bovine serum albumin. S-303-treated and untreated responder cells were co-cultured with the gamma-irradiated stimulator cells at a 1:1 ratio.

Lymphocyte proliferation was measured by addition of  $^3$ H-thymidine to the co-culture six days after the start of co-culture, harvesting cells on day 7, and measuring incorporation of  $^3$ H cpm in the cells. Cytokine production was analyzed by sandwich ELISA of culture supernatants taken 24 and 48 hours after the start of co-culture.

Analysis of proliferative ability of S-303-treated cells (Figure 16) shows that  $^3$ H-thymidine incorporation in S-303-treated, allostimulated responder cells was reduced in cells treated with 0.1  $\mu$ M S-303, and completely blocked in cells treated with S-303 concentrations of 0.2  $\mu$ M or greater. Thus, a dose-dependent inhibition of lymphocyte proliferation was observed in S-303-treated leukocytes.

Gamma interferon (IFN- $\gamma$ ) production was assessed as a measure of cytokine production by S-303-treated leukocytes. Figure 17 shows that IFN- $\gamma$  secretion was unaffected in cells treated with 0.1  $\mu$ M and 0.2  $\mu$ M S-303, in which proliferation is severely reduced and completely inhibited, respectively (See Figure 16). Cells that had been treated with 0.3  $\mu$ M S-303, in which lymphocyte proliferation is completely inhibited, (Figure 16) produced IFN- $\gamma$  at approximately 28% of control levels (Figure 17).

In summary, proliferation of allostimulated human lymphocytes, measured in a MLR, was inhibited in a dose-dependent fashion by prior treatment with S-303. Furthermore, under conditions where proliferation is completely inhibited (*e.g.*, treatment of responder cells with 0.2  $\mu$ M S-303), synthesis of IFN- $\gamma$  remained unaffected. Thus, S-303 treatment conditions can be obtained that allow one to generate lymphocytes that are incapable of proliferation upon stimulation, yet retain immunological activity.

While the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications may be practiced without departing from the spirit of the invention. Therefore the foregoing descriptions and examples should not be construed as limiting the scope of the invention.

5

CLAIMS

What is claimed is:

1. An isolated population of cells to be used for introduction into an allogeneic recipient, the cell population comprising a population of leukocytes,

5 wherein a portion of the leukocyte population is non-proliferating such that induction of graft-*versus*-host disease (GVHD) in the recipient is inhibited, and

wherein a portion of the leukocyte population retains immunological activity.

10 2. The cell population of claim 1, wherein the leukocyte population comprises T cells, NK cells and antigen-presenting cells.

3. The cell population of claim 1 wherein, upon appropriate stimulation, a portion of the leukocyte population produces cytokines.

4. The cell population of claim 3 wherein the cytokines are selected from the group consisting of IL-1 IL-2, IL-4, IFN- $\gamma$ , IL-10 and GM-CSF.

15 5. The cell population of claim 1 wherein a portion of the leukocyte population expresses a surface marker selected from the group consisting of CD2, CD28, CTLA4, CD40 ligand (gp39), CD18, CD25, CD69 (lymphocyte activation marker) and CD16/CD56, MHC Class I and Class II, CD8, CD4, CD3/TcR (T cell receptor), CD54 (ICAM -1), LFA-1 and VLA-4.

20 6. The cell population of claim 1, wherein greater than 90% of the leukocytes in the population are non-proliferating.

7. The cell population of claim 1, wherein the leukocytes have been stimulated by exposure to an antigen or a mitogen.

25 8. The cell population of claim 1 wherein the leukocytes are effective in promoting destruction of a diseased cell.

9. The cell population of claim 1 wherein the leukocytes are effective in promoting destruction of an infected cell.

10. The cell population of claim 1 wherein the leukocytes facilitate engraftment by a second population of cells.

11. The cell population of claim 10, wherein the second population of cells is selected from the group consisting of hematopoietic cells, myeloid cells, leukocytes, bone marrow cells, islet cells, hepatic cells, neuronal cells, myocardial cells, mesenchymal cells and endothelial cells.

5 12. The cell population of claim 10, wherein the second population of cells is an organ.

13. The population of claim 1 wherein the population of leukocytes comprises a first subpopulation of lymphocytes.

10 14. The population of claim 13 wherein the first subpopulation of lymphocytes comprises a second subpopulation of T-lymphocytes.

15 15. The population of claim 14 wherein the second subpopulation is obtained based on surface marker expression.

16. The population of claim 15 wherein the surface marker is selected from the group consisting of CD8, CD4, CD16 and CD56.

15 17. The population of claim 1 wherein the population of leukocytes is obtained from whole blood by a procedure selected from the group consisting of leukophoresis and red cell removal.

20 18. A method for donor leukocyte infusion, wherein the method comprises introducing a cell population according to claim 1 into a mammalian subject subsequent to a bone marrow transplant.

19. A method for enhancing engraftment of a population of transplanted cells, wherein the method comprises introducing a cell population according to claim 1 into a mammalian subject prior to the time of transplant.

25 20. A method for enhancing engraftment of a population of transplanted cells, wherein the method comprises introducing a cell population according to claim 1 into a mammalian subject at the time of transplant.

21. A method for enhancing engraftment of a population of transplanted cells, wherein the method comprises introducing a cell population according to claim 1 into a mammalian subject subsequent to the time of transplant.

22. The method according to claim 20, wherein the population of transplanted cells comprises hematopoietic stem cells.

23. The method according to claim 21, wherein the population of transplanted cells comprises hematopoietic stem cells.

5 24. A method for immune reconstitution, wherein the method comprises introducing a cell population according to claim 1 into a mammalian subject.

25. A method for adoptive immunotherapy, wherein the method comprises introducing a cell population according to claim 1 into a mammalian subject.

10 26. A method for treatment of mixed chimerism, wherein the method comprises introducing a cell population according to claim 1 into a mammalian subject.

27. A method for preparing a cell population according to claim 1, wherein the method comprises:

(a) forming an *in vitro* reaction mixture comprising a population of leukocytes and a compound capable of forming a covalent bond with a nucleic acid; and

15 (b) incubating the reaction mixture under conditions that yield a population of cells comprising a population of leukocytes;

wherein a portion of the leukocyte population is non-proliferating such that induction of graft-*versus*-host disease (GVHD) in the recipient is inhibited, and

wherein a portion of the leukocyte population retains immunological activity.

20 28. The method according to claim 27 wherein immunological activity includes destruction of a diseased cell, an infected cell or a pathogen.

29. The method according to claim 27 wherein the compound is present in an amount such that the compound forms between about 1 to about  $10^4$  adducts per  $10^8$  base pairs of genomic DNA of the leukocytes.

25 30. The method according to claim 27, wherein the compound is present in an amount such that the compound forms between about 5 to about  $10^3$  adducts per  $10^8$  base pairs of genomic DNA of the leukocytes.

31. The method according to claim 27 wherein unreacted compound is removed.

32. The method according to claim 31 wherein unreacted compound is removed by addition of a quencher to the reaction mixture.

33. A population of cells prepared according to the method of claim 27.

34. A population of cells whose properties are equivalent to those of the population according to claim 33.

35. A population of cells whose properties are equivalent to those of the population according to claim 1.

36. The method according to claim 27, wherein proliferation is inhibited in at least 90% of T-cells within the treated leukocyte population.

37. The method according to claim 27 wherein the compound comprises a nucleic acid binding moiety capable of binding non-covalently with a nucleic acid.

38. The method according to claim 27, wherein the compound comprises:

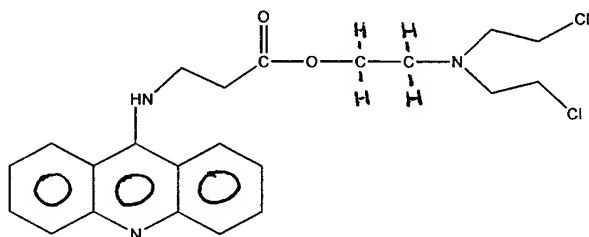
(a) a nucleic acid binding moiety;

(b) an effector moiety capable of forming a covalent bond with nucleic acid; and

(c) a frangible linker covalently linking the nucleic acid binding moiety and the effector moiety.

39. The method of claim 38, wherein the nucleic acid binding moiety is an aromatic intercalator and the effector moiety is a mustard.

40. The method according to claim 39, wherein the compound is  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester (S-303) having the formula:



and salts thereof.

41. The method of claim 27, wherein the compound is also a replication inhibitor.



42. The method of claim 27, wherein the compound is also a topoisomerase inhibitor.

43. The method of claim 42 wherein the compound is selected from the group consisting of camptothecin and daunomycin.

5 44. The method of claim 27, wherein said compound comprises a photoactivatable moiety which, upon electromagnetic stimulation, forms a covalent bond with a nucleic acid.

10 45. The method of claim 44, wherein the method further comprises exposing the reaction mixture to light to photoactivate said photoactivatable moiety, thereby resulting in formation of a covalent bond between the photoactivatable moiety and leukocyte genomic DNA.

15 46. The method of claim 45, wherein the compound is selected from the group consisting of furocoumarins, actinomycins, anthracyclines, anthramycins, benzodipyrones, fluorenes, fluorenones, monostral fats blue, norphillin A, organic dyes; phenanthridines, phenazathionium salts, phenazines, phenothiazines, phenylazides, quinolines, thiaxanthenones, acridines and ellipticenes.

47. The method of claim 46, wherein the furocoumarin is a psoralen.

20 48. The method of claim 47, wherein the psoralen is selected from the group consisting of PAP, 8-methoxy psoralen (8-MOP), 4'-aminomethyl 4,5',8-trimethylpsoralen (AMT), 5-methoxy psoralen, and trioxalen 4, 5' 8-trimethylpsoralen.

49. The method of claim 47, wherein the reaction mixture is exposed to ultraviolet light having a wavelength in the range of 200 to 450 nm.

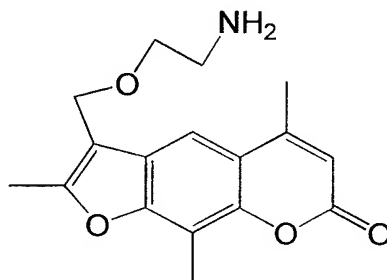
50. The method of claim 49, wherein the reaction mixture is exposed to ultraviolet light having a wavelength in the range of 320 to 400 nm.

25 51. The method of claim 50, wherein the reaction mixture is exposed to ultraviolet light at a dosage of between  $10^{-3}$  to  $100 \text{ J/cm}^2$ .

52. The method of claim 51, wherein the reaction mixture is exposed to the ultraviolet light for a period of 1 second to 60 minutes.

30 53. The method of claim 52, wherein the psoralen is present at a concentration in the range of  $10^{-4}$  to  $150 \mu\text{M}$ .

54. The method of claim 53, wherein said psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen (S-59) having the formula:



and salts thereof.

5 55. The method of claim 54, wherein the S-59 is at a concentration in the range of  $10^{-3}$  to  $150 \mu\text{M}$ .

56. The method of claim 55, wherein the reaction mixture is exposed to ultraviolet light having a wavelength in the range of 200 to 450 nm.

10 57. The method of claim 56, wherein the reaction mixture is exposed to ultraviolet light having a wavelength in the range of 320 to 400 nm.

58. The method of claim 57, wherein the reaction mixture is exposed to ultraviolet light at a dosage of between  $10^{-3}$  to  $100 \text{ J/cm}^2$ .

59. The method of claim 58, wherein the reaction mixture is exposed to ultraviolet light at a dosage of  $3 \text{ J/cm}^2$ .

15 60. The method of claim 58, wherein the reaction mixture is exposed to the ultraviolet light for a period of between 1 second to 60 minutes.

61. The method of claim 60, wherein the reaction mixture is exposed to the ultraviolet light for a period of approximately 1 minute.

20 62. The method of claim 61, wherein the population of leukocytes is at a cell density of  $10$  to  $10^9$  cells per mL.

63. The method of claim 62, wherein the population of leukocytes is at a cell density of  $2 \times 10^6$  cells per mL.

64. A population of cells produced according to the method of claim 38.

65. A population of cells produced according to the method of claim 40.

66. A population of cells produced according to the method of claim 41.

67. A population of cells produced according to the method of claim 46.

68. A population of cells produced according to the method of claim 54.

69. A population of cells produced according to the method of claim 59.

5 70. A population of cells produced according to the method of claim 63.

71. A method for promoting destruction of a diseased cell or a pathogen, comprising mixing the leukocyte population of claim 1 with a population of allogeneic cells containing the diseased cell or pathogen.

10 72. A method for promoting destruction of a diseased cell or a pathogen, comprising mixing the leukocyte population of claim 70 with a population of allogeneic cells containing the diseased cell or pathogen.

73. The method of claim 72, wherein the diseased cell is a cancerous cell.

15 74. The method of claim 73, wherein the cancerous cell is selected from the group consisting of Chronic Myelogenous Leukemia (CML) cell, Chronic myelomonocytic Leukemia (CmML) cell, Chronic Lymphocytic Leukemia (CLL) cell, Acute Myelogenous Leukemia (AML) cell, Acute Lymphoblastic Leukemia (ALL) cell, multiple myeloma (MM) cell, Hodgkin's lymphoma cell and non-Hodgkin's lymphoma cell.

20 75. The method of claim 74, wherein the cancerous cell is a Chronic Myelogenous Leukemia cell.

76. The method of claim 74, wherein the cancerous cell is a multiple myeloma cell.

25 77. The method of claim 73, wherein the cancerous cell is selected from the group consisting of breast cancerous cell, lung cancerous cell, ovarian cancerous cell, testicular cancerous cell, prostate cancerous cell, colon cancerous cell, melanoma cell, renal carcinoma cell, neuroblastoma cell, head tumor cell and neck tumor cell.

78. The method of claim 71, wherein the diseased cell is an infected cell.

79. The method of claim 78, wherein the infected cell is infected with a virus.

80. The method of claim 79, wherein the virus is selected from the group consisting of cytomegalovirus (CMV), Epstein Barr virus (EBV), Adenovirus (Ad) and Kaposi's Sarcoma associated Herpes virus.

5 81. The method of claim 71, wherein the leukocyte population is mixed with the population of allogeneic cells of a mammalian host *in vivo* by donor leukocyte infusion into said host.

82. The method of claim 81, wherein the mammalian host is suffering from relapse from leukemia or multiple myeloma post bone marrow transplantation.

10 83. The method of claim 71, wherein the leukocyte population has been stimulated to expand the number of cytotoxic T cells specific to an antigen of the diseased cell or pathogen.

84. The method of claim 83, wherein the stimulation is performed *in vivo* prior to isolation of the leukocyte population from the donor.

15 85. The method of claim 84, wherein the stimulation is performed by vaccination of the leukocyte donor with the antigen.

86. The method of claim 85, wherein the diseased cell is a CML cell and the leukocyte donor is vaccinated with a *bcr-abl* antigen of the CML cell.

87. The method of claim 85, wherein the diseased cell is a multiple myeloma cell and the leukocyte donor is vaccinated with the idiotype antigen of the myeloma cell.

20 88. The method of claim 83, wherein the stimulation is performed *ex vivo*.

89. The method of claim 83, wherein stimulation results from contact between the leukocyte population and a diseased cell.

25 90. The method of claim 83, wherein stimulation results from contact between the leukocyte population and an antigen-presenting cell, wherein the antigen-presenting cell has been exposed to the diseased cell.

91. The method of claim 83, wherein stimulation results from contact between the leukocyte population and an antigen-presenting cell, wherein the antigen-presenting cell has been exposed to an antigen of the diseased cell.

92. A method for preparing a cell population according to claim 1, wherein the method comprises:

(a) forming an *in vitro* reaction mixture comprising a population of leukocytes and a replication-inhibitory compound; and

5 (b) incubating the reaction mixture under conditions that yield a population of cells comprising a population of leukocytes wherein a portion of the leukocyte population is non-proliferating such that induction of graft-*versus*-host disease (GVHD) in the recipient is inhibited, and wherein a portion of the leukocyte population retains immunological activity.

10 93. The method of claim 92, wherein the compound is a topoisomerase inhibitor.

94. The method of claim 93 wherein the compound is selected from the group consisting of camptothecin and daunomycin.

15 95. A method for preparing a treated leukocyte population wherein the leukocyte population as a whole is non-proliferating and incapable of eliciting graft-*versus*-host disease (GVHD) in an allogeneic host, comprising the steps of:

i) providing a sample of a purified population of leukocytes; and  
ii) combining the sample of leukocytes with a compound capable of forming a covalent bond with a nucleic acid, in an amount such that the compound forms  
20 about 1 to 10<sup>4</sup> adducts per 10<sup>8</sup> base pairs of genomic DNA of the leukocytes,  
thereby inhibiting proliferation but maintaining the effectiveness of the leukocyte population to promote destruction of a diseased cell or a pathogen.

25 96. A method for preparing stimulator cells for a mixed lymphocyte reaction wherein a population of cells comprising a population of leukocytes is treated according to the method of claim 27 and the treated cell population is used as stimulator cells.

97. A method for determining whether proliferation is required for a cell function comprising the steps of:

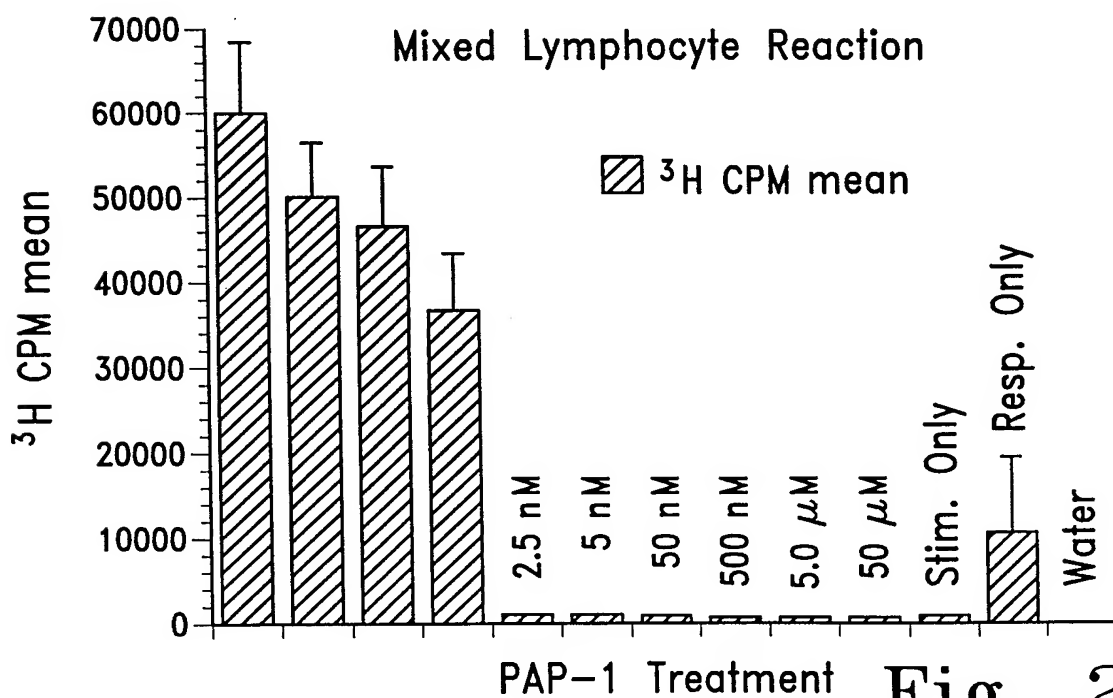
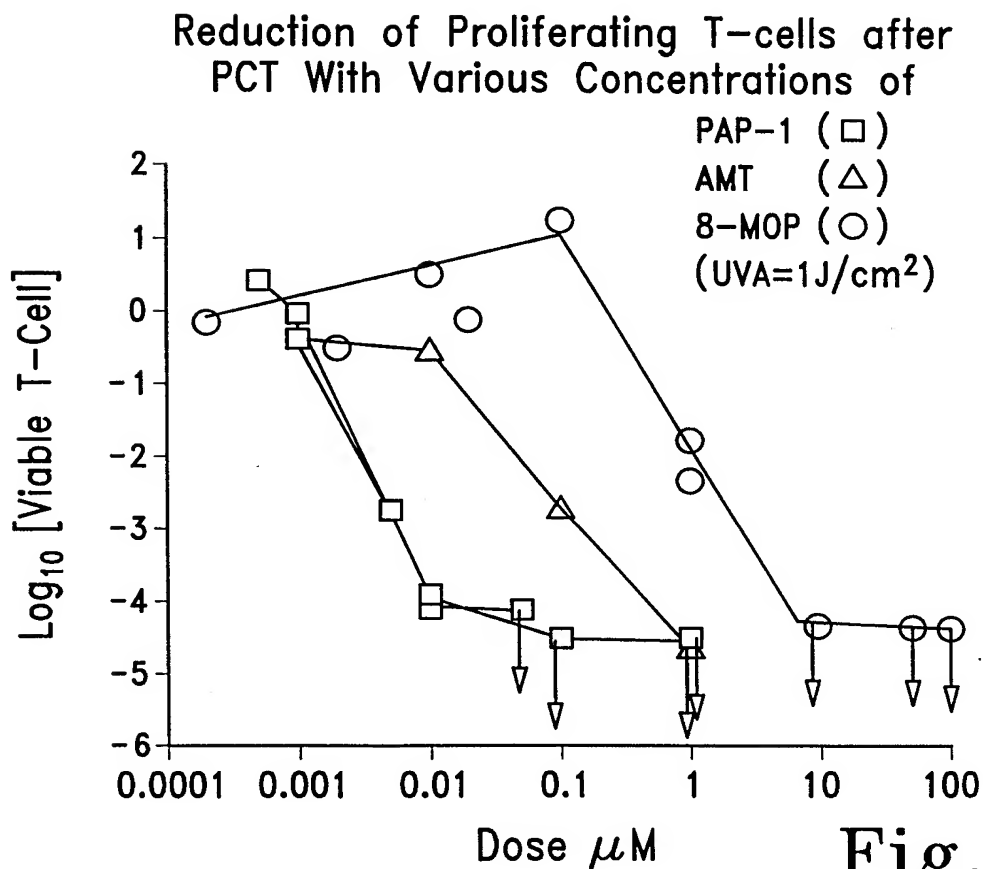
(a) forming an *in vitro* reaction mixture comprising a population of cells and a compound capable of forming a covalent bond with a nucleic acid;

(b) incubating the reaction mixture under conditions that yield a population of cells that are incapable of DNA synthesis but capable of RNA and protein biosynthesis; and

observing the cell population to determine whether the cell function is carried out.

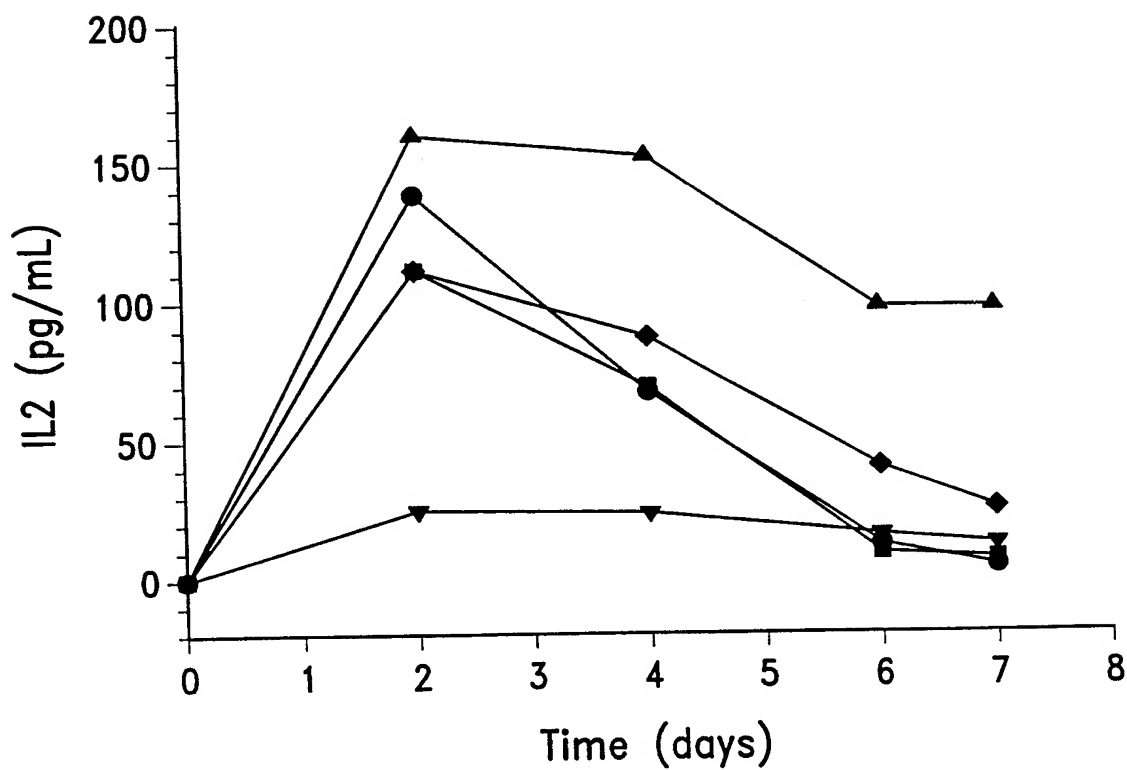
5           98.     The method of claim 97 wherein the cell function is differentiation.

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IL2 Production in an MLR  
by Photochemically Treated  
Effector Cells



PAP-1 concentration

- 0  $\mu\text{M}$
- 0.00005  $\mu\text{M}$
- ◆— 0.0005  $\mu\text{M}$
- ▲— 0.005  $\mu\text{M}$
- ▼— Stimulators

Fig. 3



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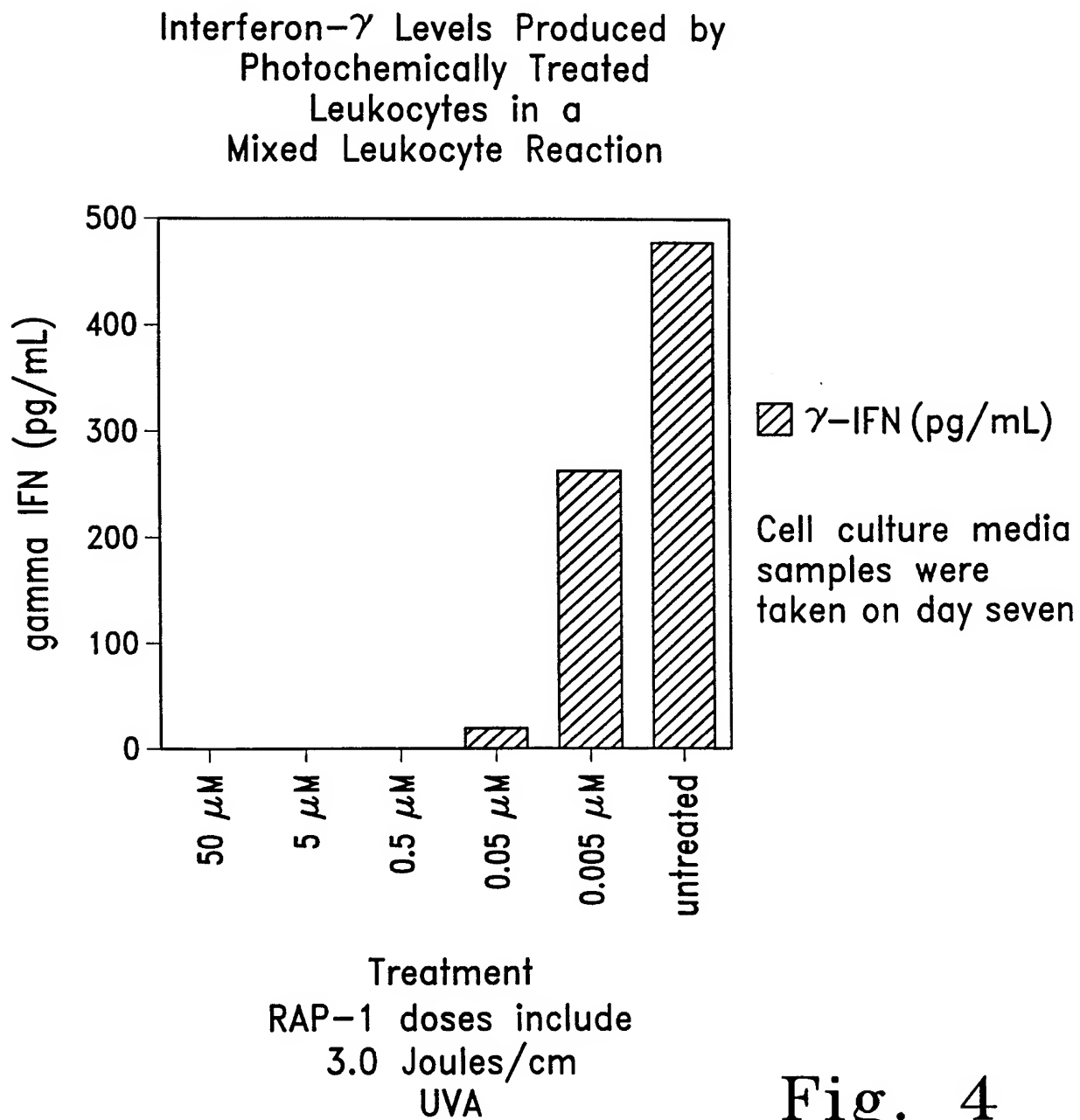


Fig. 4

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IL-8 Levels in PC during storage after PCT with PAP-1  
 $0.5\text{J}/\text{cm}^2$

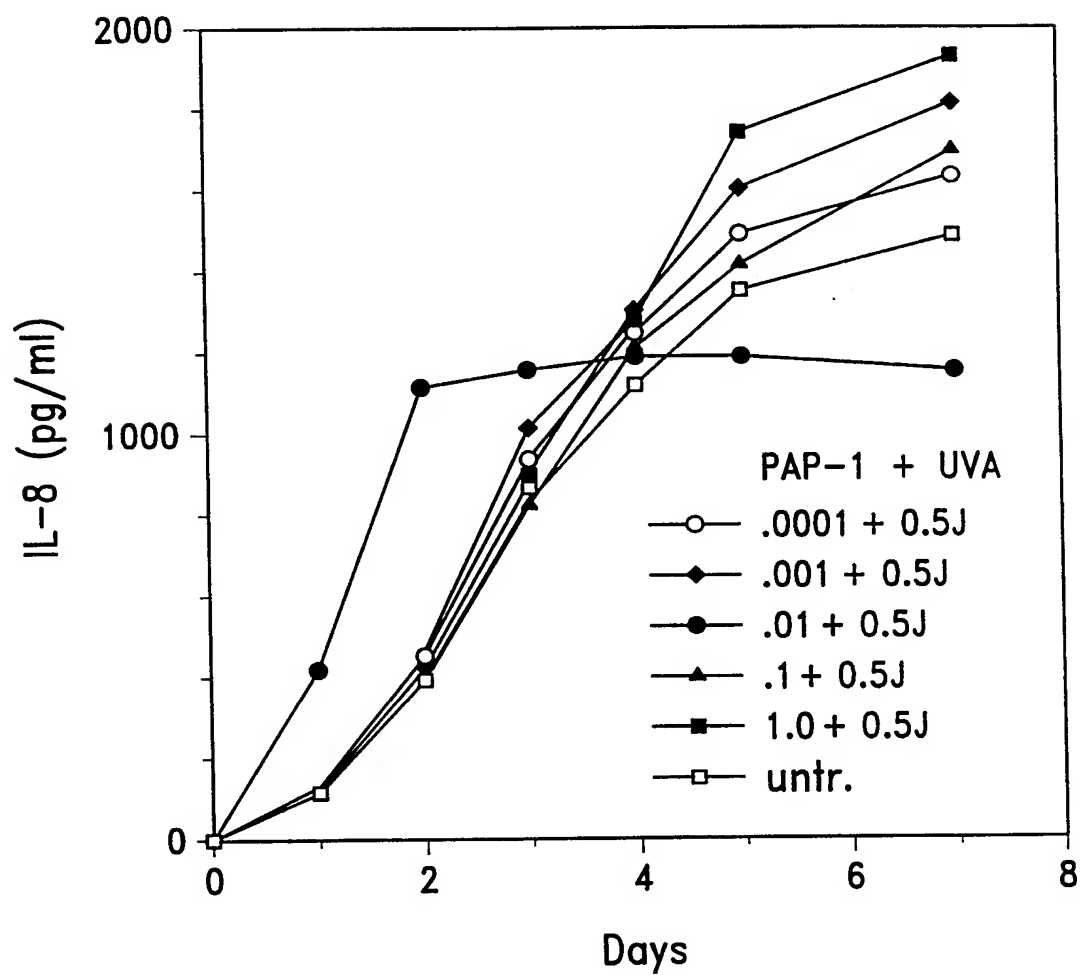


Fig. 5

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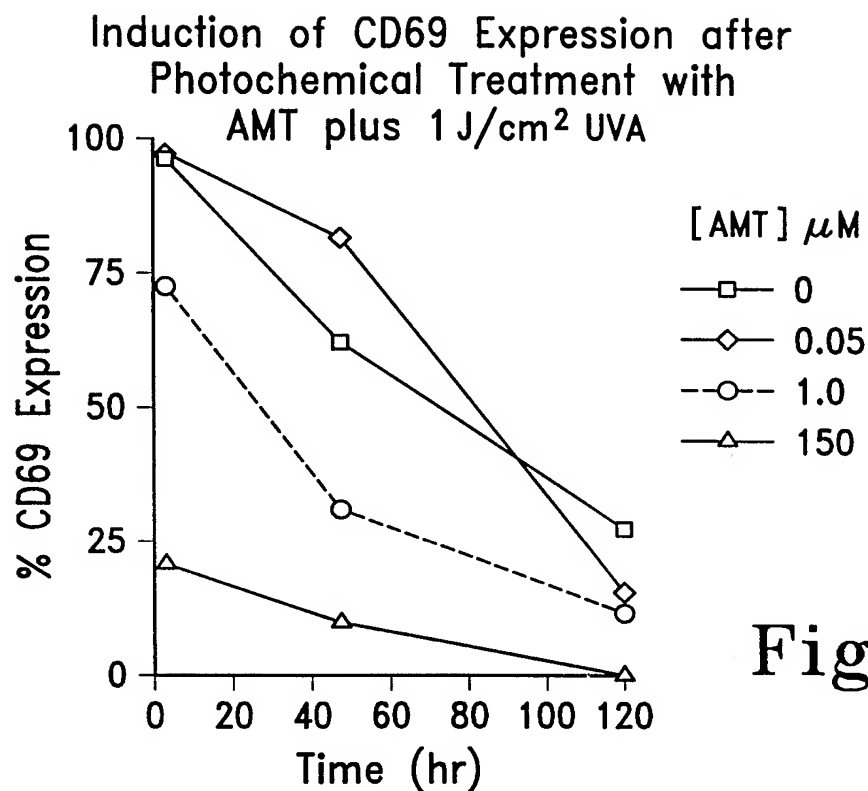


Fig. 6

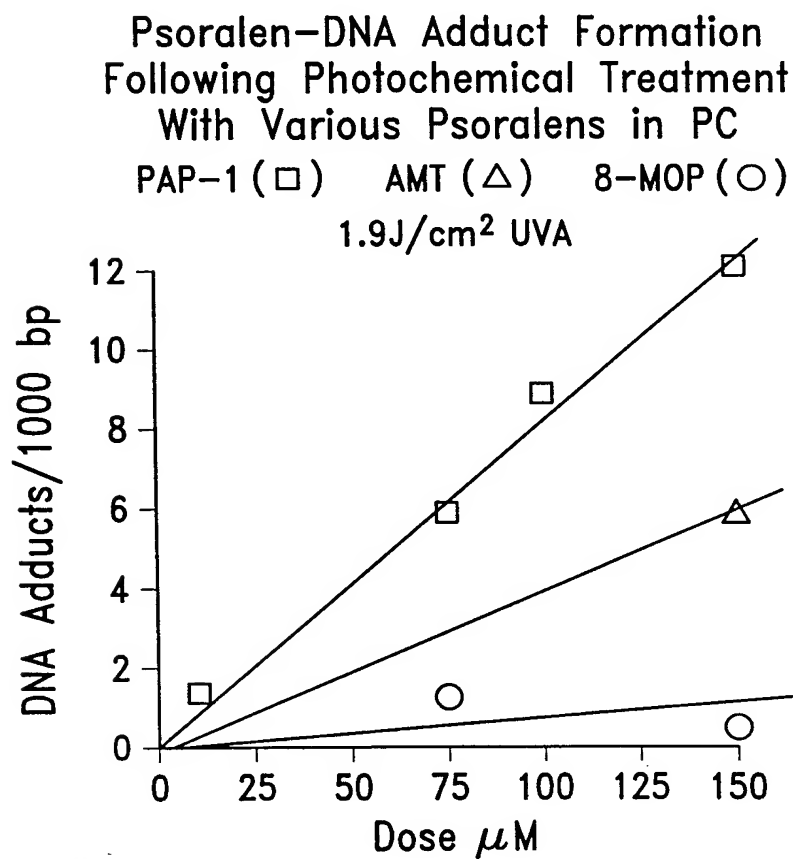


Fig. 7

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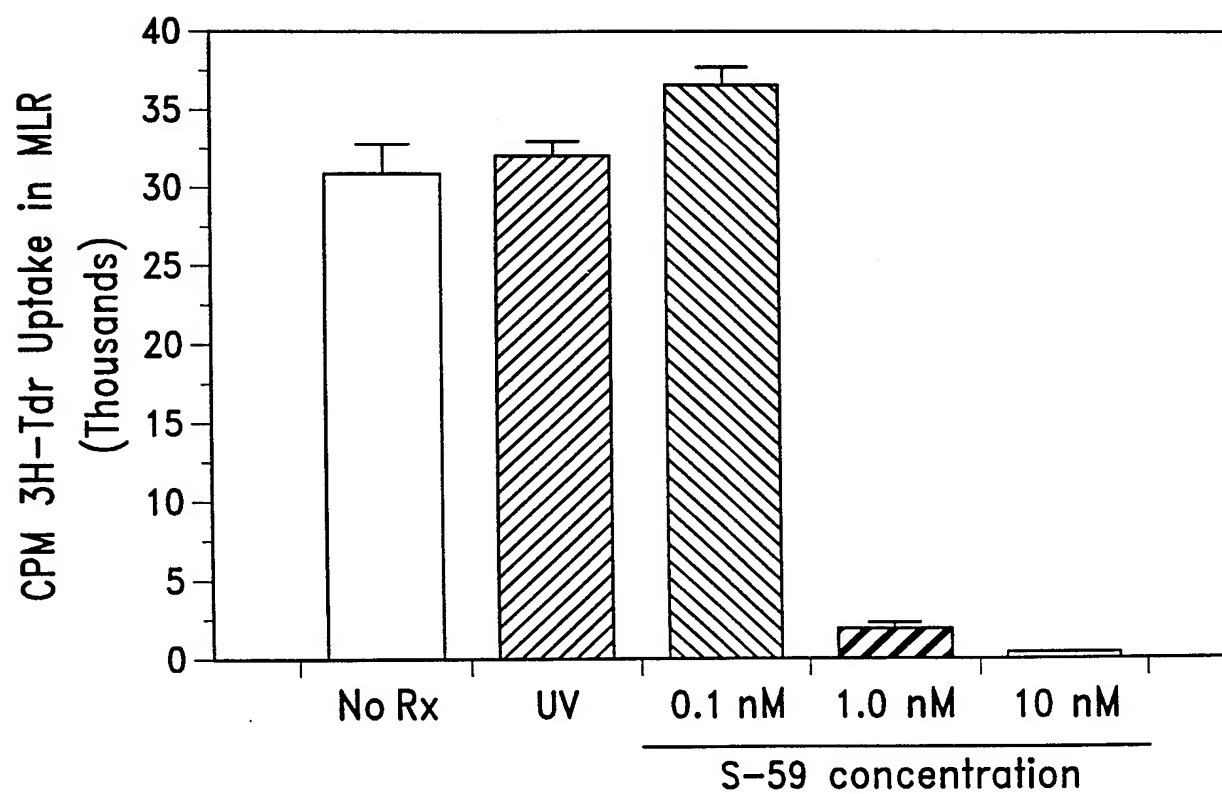


Fig. 8

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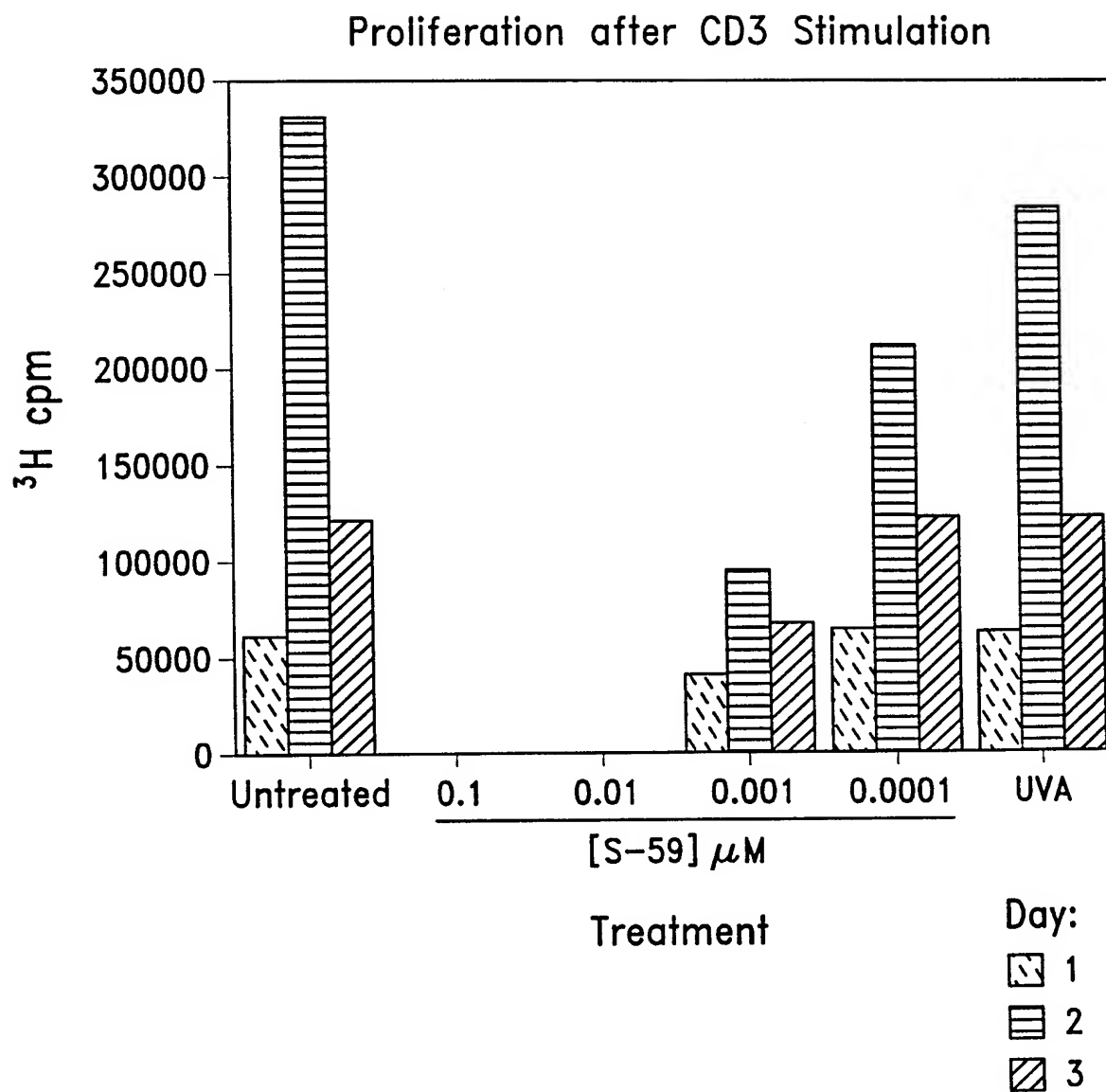


Fig. 9

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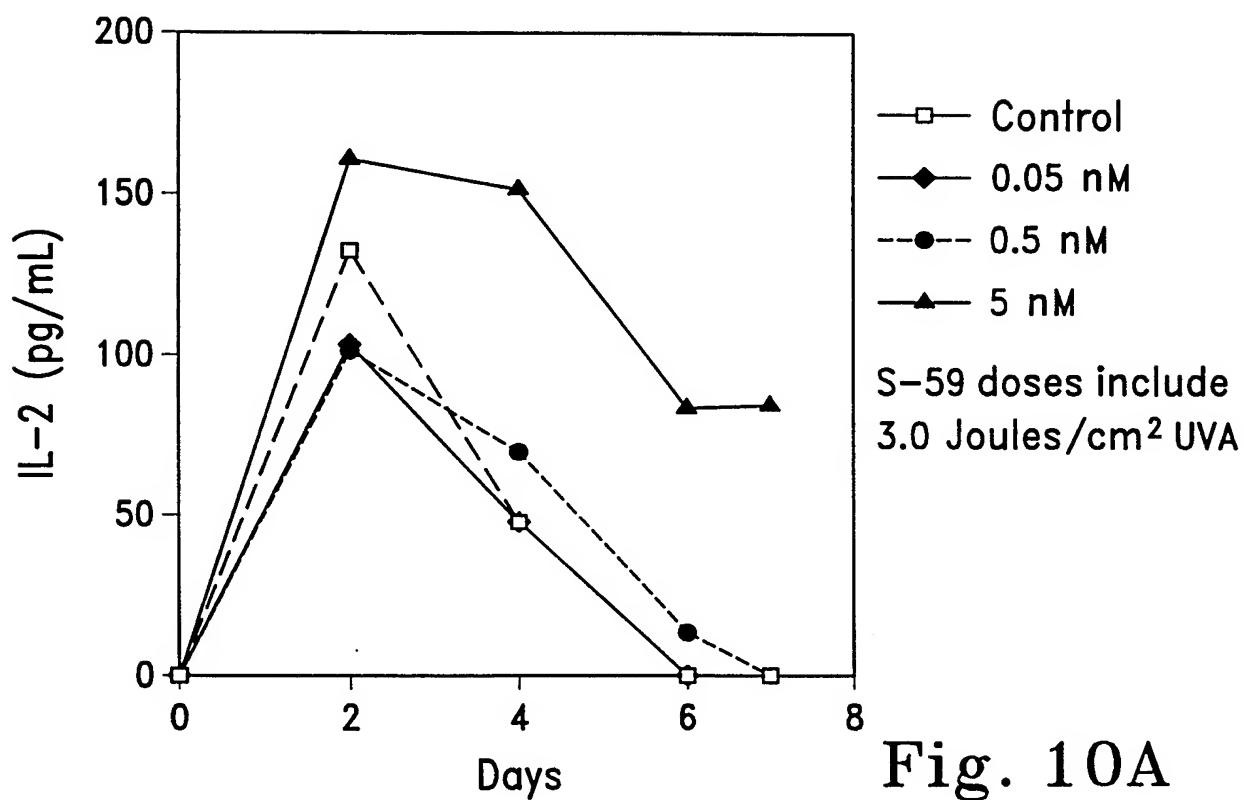


Fig. 10A

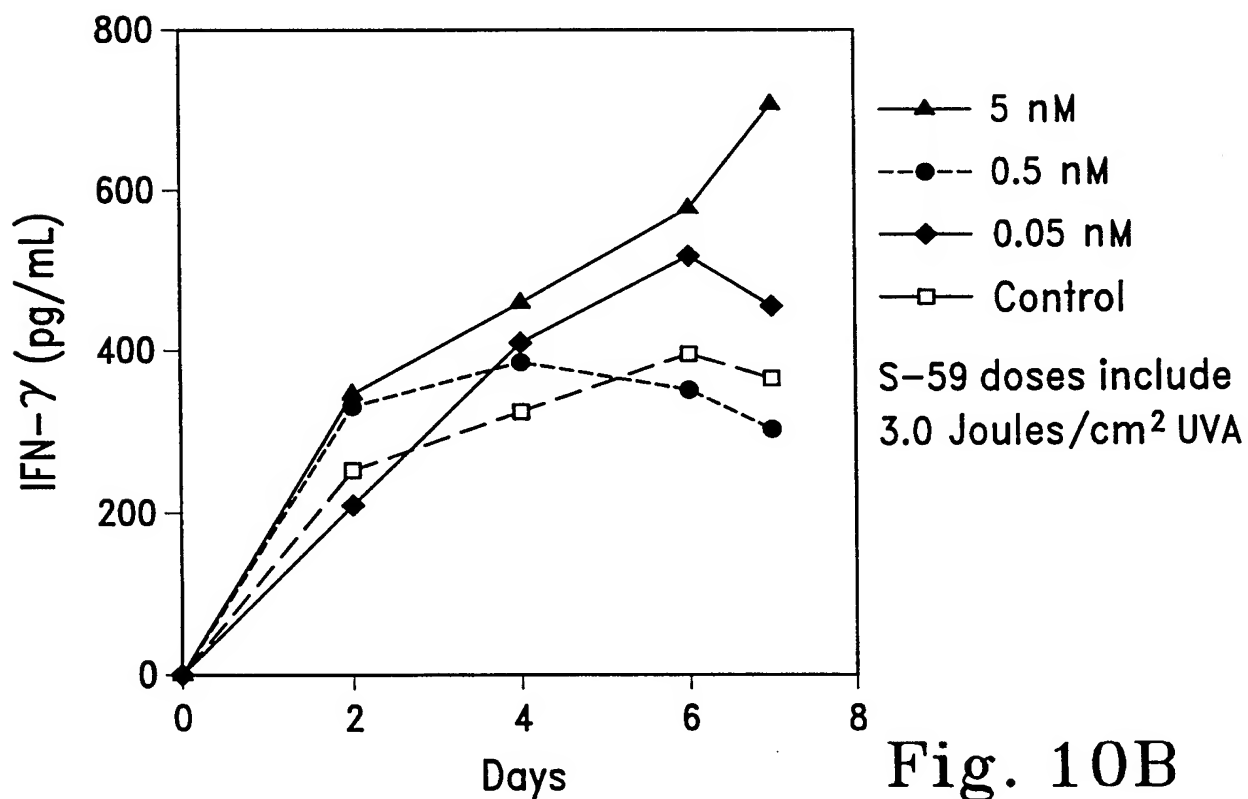
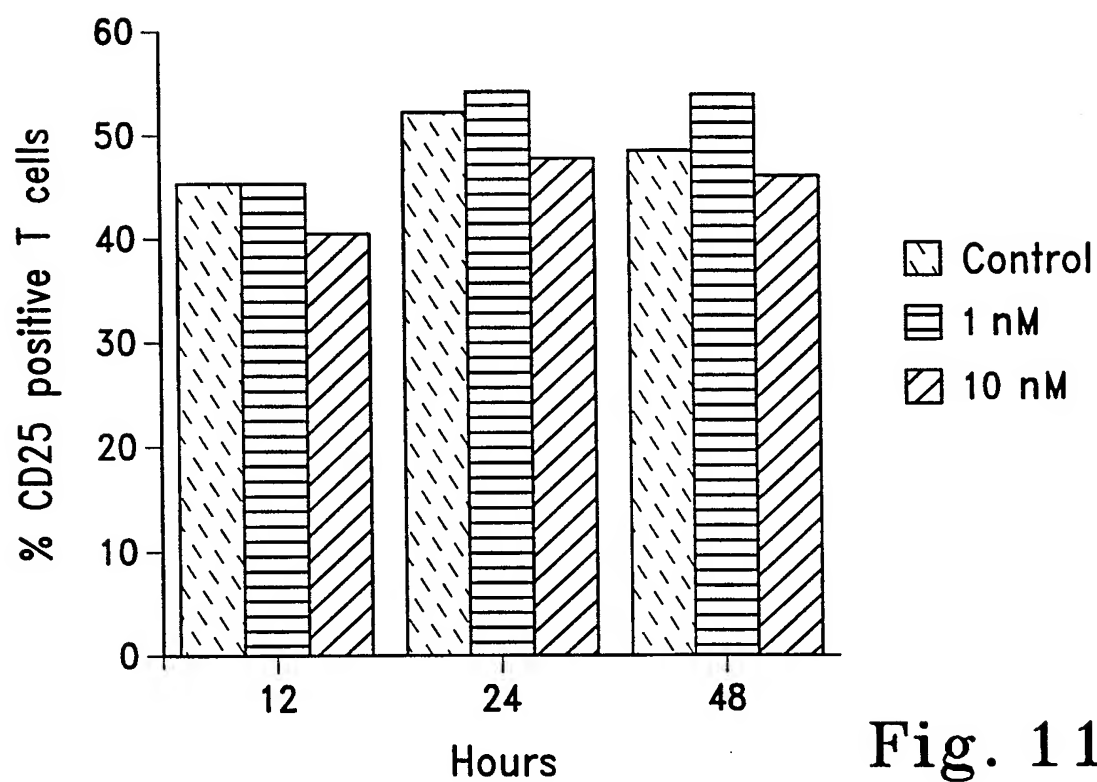
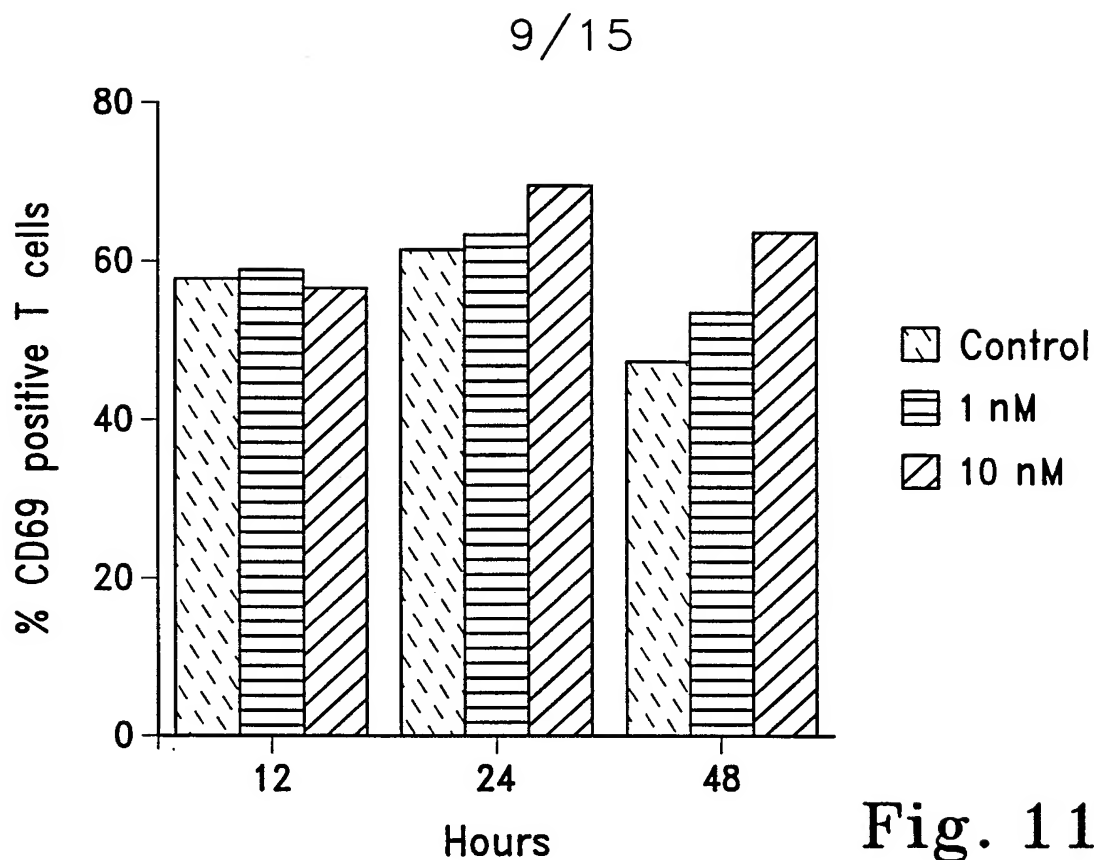


Fig. 10B



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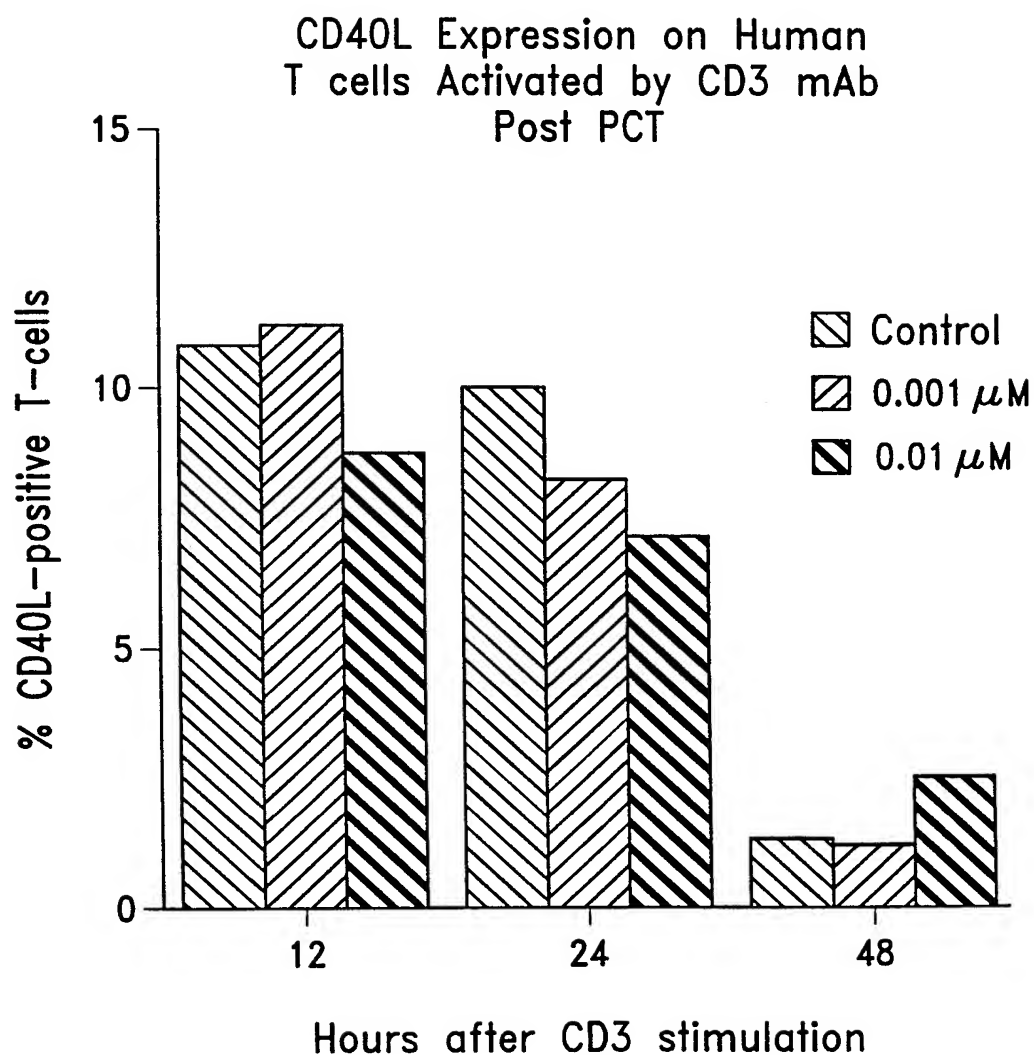


Fig. 12



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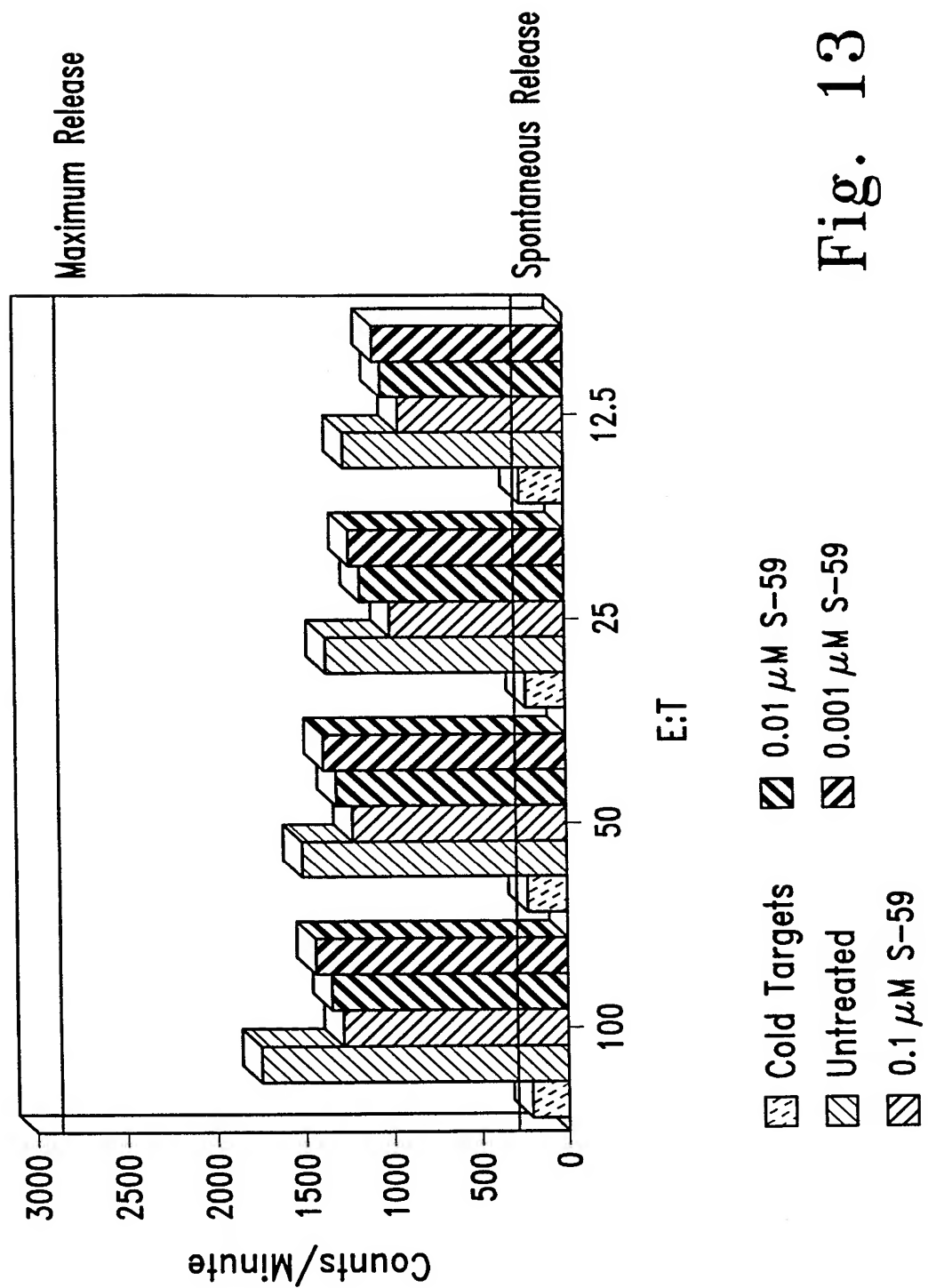
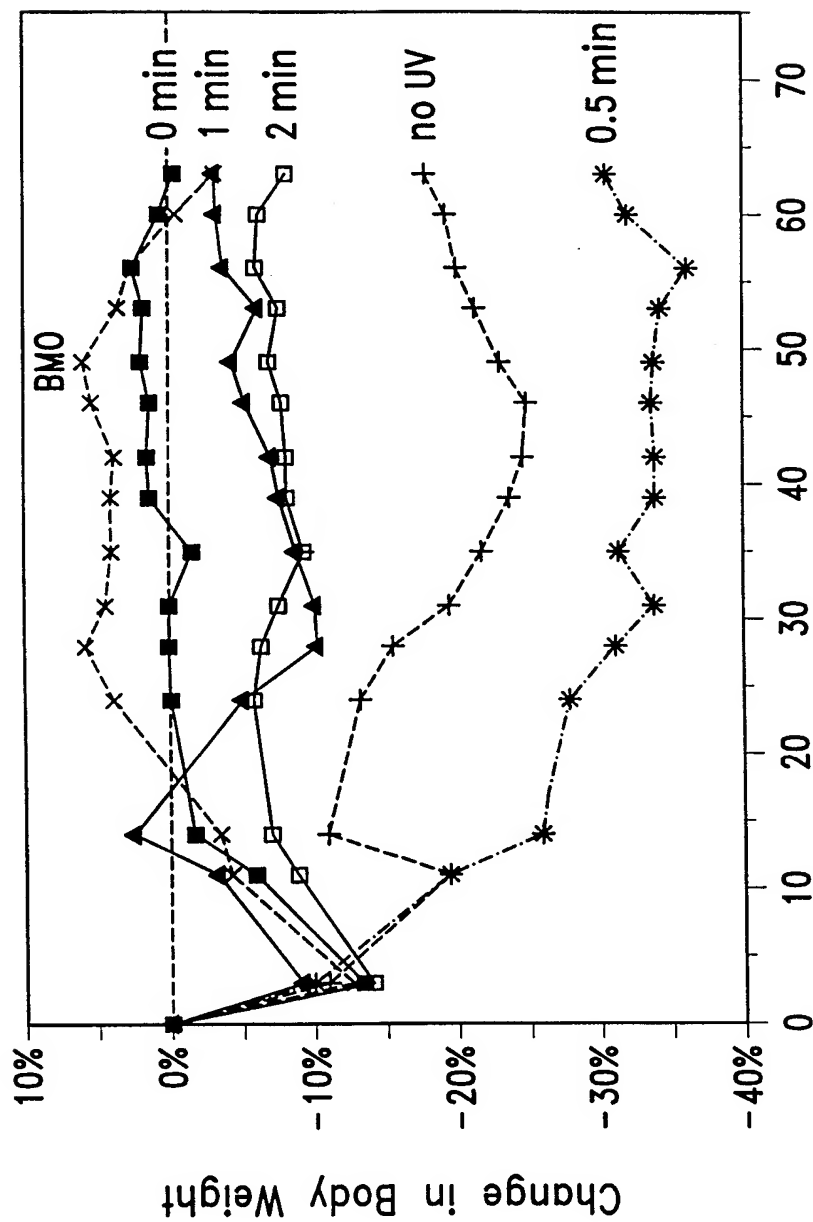


Fig. 13

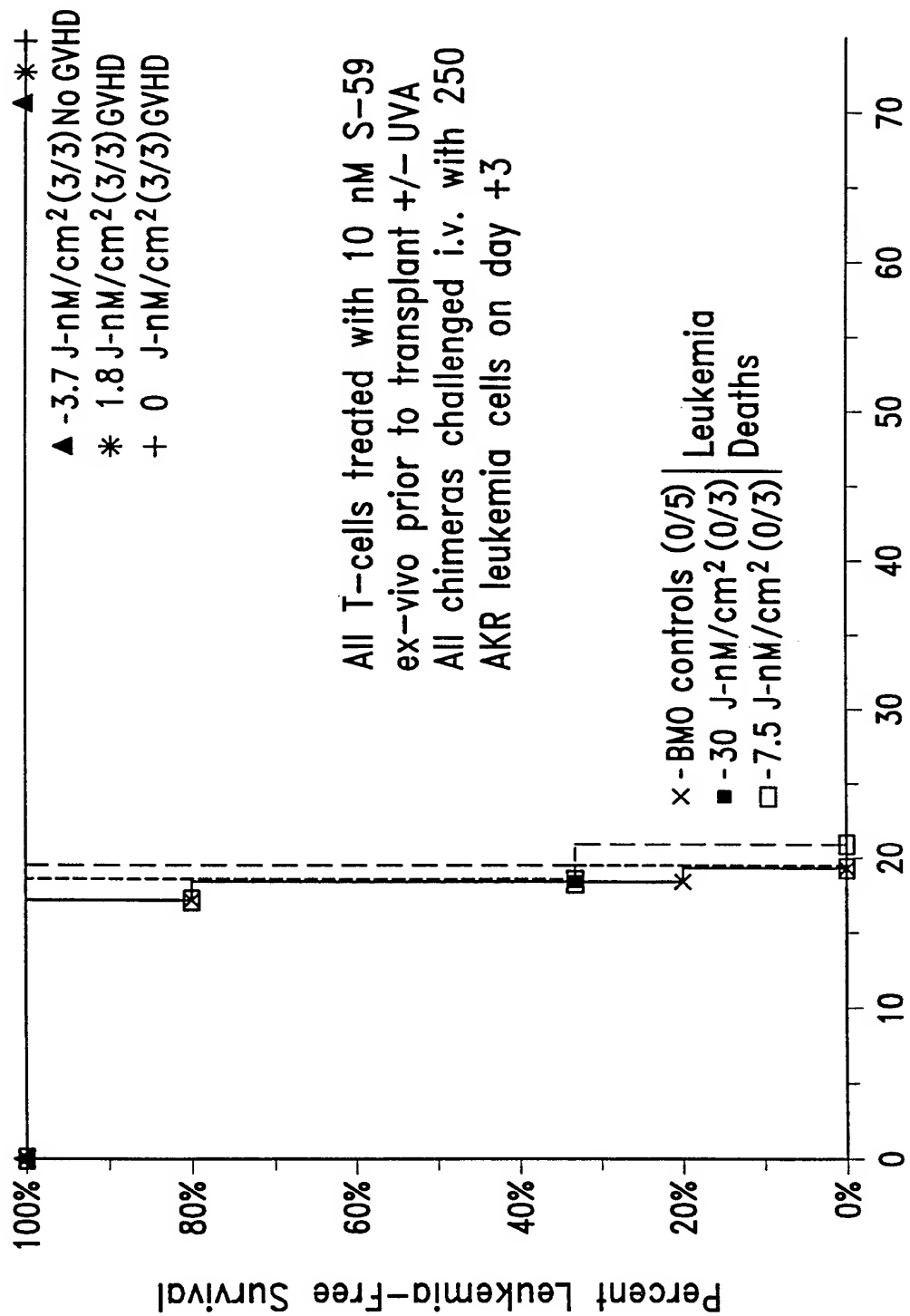
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Days after Transplant

Fig. 14

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Days after Transplant

Fig. 15

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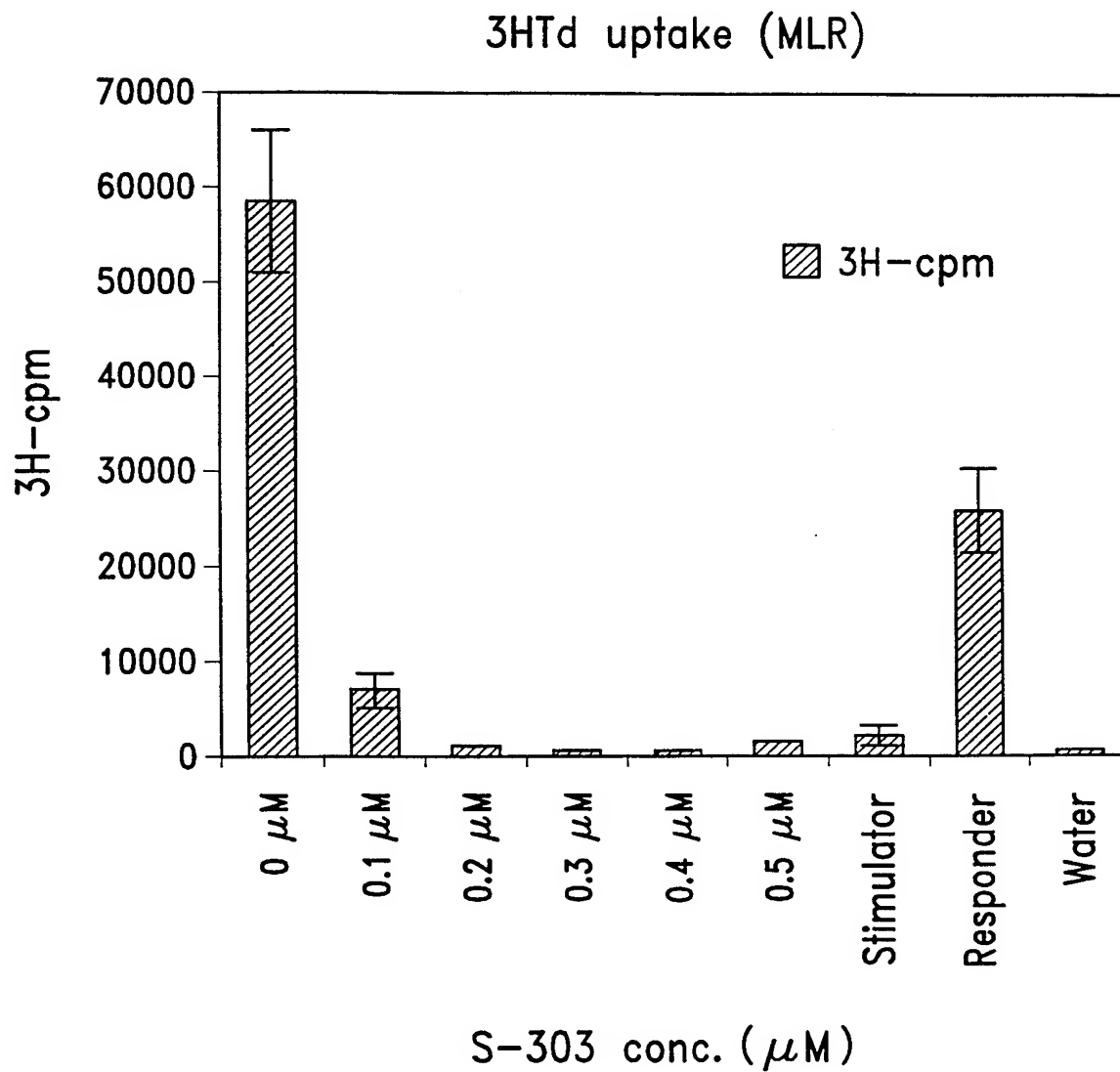


Fig. 16

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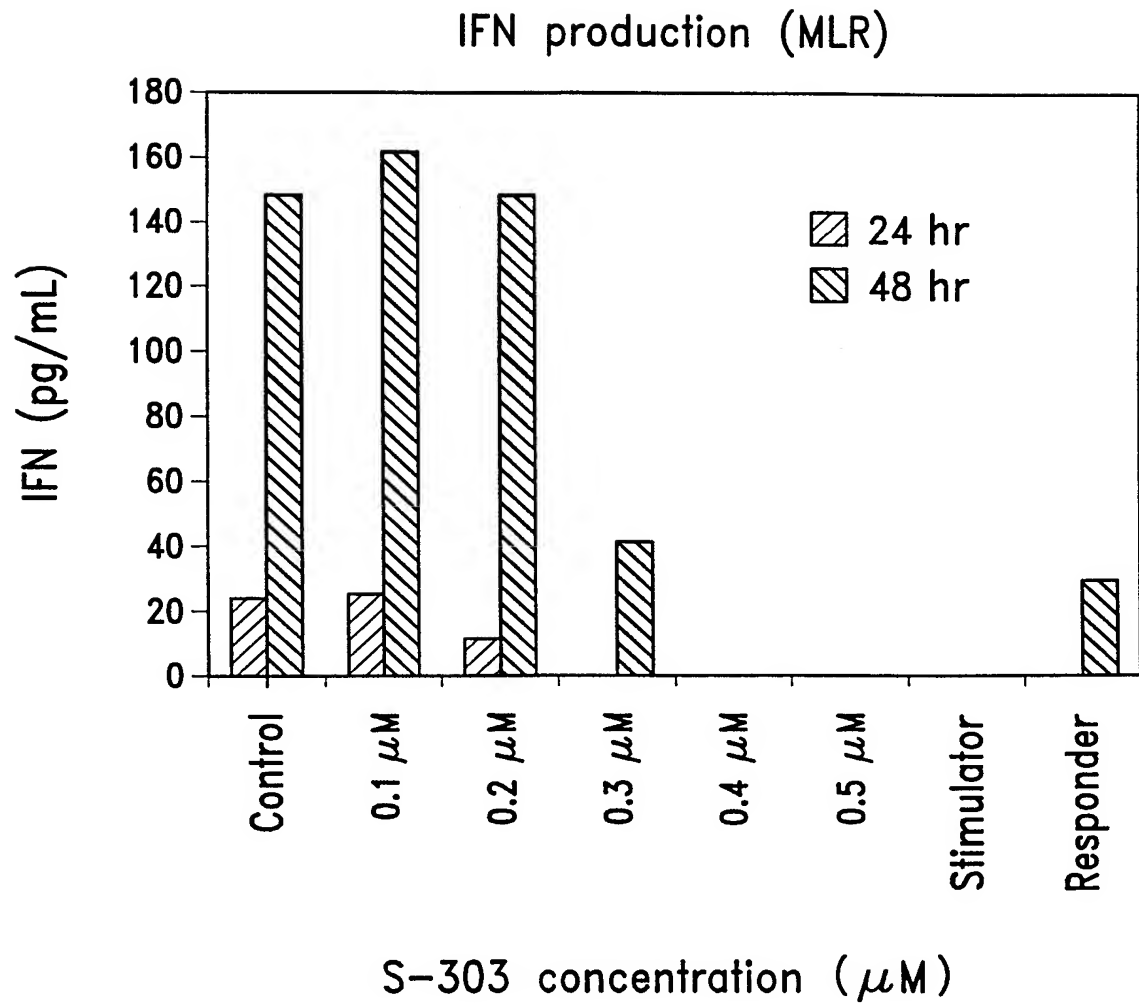


Fig. 17



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C12N 5/06, 15/01, A61K 41/00		A3	(11) International Publication Number: <b>WO 99/03976</b>
			(43) International Publication Date: 28 January 1999 (28.01.99)
(21) International Application Number: PCT/US98/15067 (22) International Filing Date: 21 July 1998 (21.07.98) (30) Priority Data: 60/053,599                      21 July 1997 (21.07.97)                      US 09/119,707                      20 July 1998 (20.07.98)                      US (71) Applicant (for all designated States except US): CERUS CORPORATION [US/US]; 2525 Stanwell Drive, Concord, CA 94520 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GREENMAN, William, M. [US/US]; 3834 23rd Street, San Francisco, CA 94114 (US). GRASS, Joshua, A. [US/US]; 3187 Ida Drive, Concord, CA 94519 (US). TALIB, Sohel [US/US]; 404 Camberly Way, Redwood City, CA 94061 (US). STASSINOPOULOS, Adonis [GR/US]; 7065 York Court, Dublin, CA 94568 (US). HEI, Derek, J. [US/US]; 4405 Smoke Tree Court, Concord, CA 94521 (US). HEARST, John, E. [US/US]; 101 Southhampton Street, Berkeley, CA 94717 (US). (74) Agents: BRENNAN, Sean, M. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  (88) Date of publication of the international search report: 27 May 1999 (27.05.99)	
(54) Title: METHOD OF TREATING LEUKOCYTES, LEUKOCYTE COMPOSITIONS AND METHODS OF USE THEREOF			
(57) Abstract			
<p>The invention provides methods and compositions for treating leukocytes to arrest proliferation of the leukocytes and render them ineffective in eliciting graft-versus-host disease (GVHD), but effective to enhance engraftment of allogeneic donor cells and promote destruction of diseased cells or pathogens. Leukocyte compositions and methods of use of these compositions in alleviating disease, facilitating various types of immune reconstitution and immunotherapy, and enhancing engraftment of allogeneic donor cells, are also provided.</p>			

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# INTERNATIONAL SEARCH REPORT

national Application No  
PCT/US 98/15067

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N5/06 C12N15/01 A61K41/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category <sup>2</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 96 39820 A (CERUS CORPORATION) 19 December 1996</p> <p>see abstract see page 1, line 10 - line 17 see page 11, line 25 - page 12, line 5 see page 13, line 22 - page 15, line 20 see page 15, line 31 - page 16, line 36 see page 18, line 7 - page 19, line 3 see page 20, line 25 - page 21, line 9 see page 22, line 4 - page 30, line 16 see page 31, line 1 - line 30 see page 39, line 20 - page 40, line 36 see page 62, line 19 - page 64, line 30 see claims 1,17-25,28,34-36,38 -/--</p>	<p>1,10,11, 13,14, 27,35, 36, 44-70, 81,82, 92,95</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

<sup>3</sup> Special categories of cited documents :

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Date of the actual completion of the international search

18 March 1999

Date of mailing of the international search report

29/03/1999

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NL - 2280 HV Rijswijk  
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/15067

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	<p>see claims 42-44,60-67  see examples 14,15,22,23  ---</p> <p>US 5 651 993 A (EDELSON RICHARD L ET AL)  29 July 1997</p> <p>see abstract  see column 3, line 60 - line 67  see column 4, line 15 - line 39  see column 5, line 16 - line 47  see column 5, line 59 - line 65  see column 9, line 45 - column 10, line 13  see examples 1-4  ---</p>	<p>1,2,5,  7-16,  27-29,  32,35,  36,  44-71,  81-83,  89-91</p>
X	<p>EP 0 284 409 A (THERAKOS INC)  28 September 1988</p>	<p>1-3,7,  10-16,  27,  35-41,  44-49,  67,69,  71-73,  92,95</p>
A	<p>see abstract  see page 5, line 23 - line 62; example 2  ---</p>	<p>1-4,7,  13-15</p>
X	<p>KOZENITZKY L. ET AL.: "Immunomodulatory effects of AS101 on interleukin-2 production and T-lymphocyte function of lymphocytes treated with psoralens and ultraviolet A"  PHOTODERMATOLOGY, PHOTOIMMUNOLOGY &amp; PHOTOMEDICINE,  vol. 9, no. 1, 1992, pages 24-28,  XP002094960  see abstract  ---</p>	<p>1,10,  13-16,  26-30,  33,  36-71,  81,82,  92,95-98</p>
A	<p>ULLRICH S.E.: "Photoinactivation of t-cell function with psoralen and uVA radiation suppresses the induction of experimental murine graf-versus-host disease accross Major Histocompatibility barriers"  JOURNAL OF INVESTIGATIVE DERMATOLOGY,  vol. 96, no. 3, 1991, pages 303-308,  XP002094961  see the whole document  ---</p>	<p>1,10,  13-16,  26-30,  33,  36-71,  81,82,  92,95-98</p>

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/15067

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>HALSTENSEN T., BRANDTZAEG P.: "Activated T lymphocytes in the celiac lesion: Non proliferative activation (CD25) of CD24 positive alpha/beta cells in the lamina propria but proliferation (Ki-67) of alpha/beta and gamma/delta cells in the epithelium "</p> <p>EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 23, no. 2, 1993, pages 505-510, XP002094962 see the whole document</p> <p style="text-align: center;">---</p>	1,2,5
X	<p>GAUS H. ET AL.: "Superantigen-induced anergy of Vbeta8sup + CD4sup + T cells induces functional but non-proliferative T cells in vivo"</p> <p>IMMUNOLOGY, vol. 83, no. 3, 1994, pages 333-340, XP002094963 gb see the whole document</p> <p style="text-align: center;">---</p>	1,2,5
A	<p>WINKELSTEIN A.: "Murine B-lymphocyte colony formation: the effects of cyclophosphamide and azathiopirine"</p> <p>IMMUNOLOGY, vol. 46, - 1982 pages 827-832, XP002094965 gb see abstract see discussion</p> <p style="text-align: center;">---</p>	1,2
A	<p>MONTERO R. ET AL.: "AS-101: a modulator of in vitro T-cel proliferation"</p> <p>ANTI-CANCER DRUGS, vol. 4, no. 3, 1993, pages 351-354, XP002094966 see the whole document</p> <p style="text-align: center;">---</p>	1,2
X,P	<p>GRASS J.A. ET AL.: "Inactivation of leucocytes in platelet concentrates by photochemical treatment with psoralen plus UVA"</p> <p>BLOOD, vol. 91, no. 6, 1998, pages 2180-2188, XP002094967 see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-98

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/15067

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRASS J. ET AL.: "Prevention of transfusion-associated graft versus host disease (TA-GVHD) by photochemical treatment" BLOOD, vol. 88, no. 10 Suppl.1, 1996, page 627a XP002094968 see abstract ---	1-35, 92-98
X	GRASS J. ET AL.: "Inactivation of T-cells with psoralens and UVA in human platelet concentrates" BLOOD, vol. 86, no. 10 suppl.1, - 1995 page 542a XP002094969 see abstract -----	1-35, 92-98

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 15067

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18, 24-26  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim(s) 18, 24-26  
is(are) directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☒ Claims Nos.: 97-98  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 98 /15067

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 97-98

A search for claims 97 and 98, relating to a method to verify whether proliferation is needed by the cells for their other functions, namely differentiation, was restricted to the embodiments presented in the examples and in the previous claims, since the subject matter of claims 97 and 98 would otherwise be far beyond the specific scope of the invention (see Art.6 PCT and PCT Search Guidelines III.3.7).

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/15067

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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